FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY 'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

REG 710-A-US

U.S. APPLICATION NO. (If known, see 37 CFR 1 5

NOT **10/009852** KNOWN

INTERNATIONAL APPLICATION NO. PCT/US00/14142	INTERNATIONAL FILING DATE May 23, 2000	PRIORITY DATE CLAIMED June 8, 1999

TITLE OF INVENTION   MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF

APPLICANT(S) FOR DO/EO/US
Nicholas J. Papadopoulos, Samuel Davis, and George D. Yancopoulos

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.

4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [X] has been previously submitted under 35 U.S.C. 154(d)(4).

7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] An English lanuage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.

14. [ ] A SECOND or SUBSEQUENT preliminary amendment.

15. [ ] A substitute specification.

16. [ ] A change of power of attorney and/or address letter.

17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.

18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).

19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

20. [X] Other items or information: Express Mail Label No. ET712522493US dated December 6, 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Not Yet Known **10/009852**	PCT/US00/14142	REG 710-A-US

21. [X] The following fees are submitted:

	CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO........... $1040.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =			$	740.00
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	154 - 20 =	134	x $18.00	$	2,412.
Independent claims	5 - 3 =	2	x $84.00	$	168.
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$	280.
TOTAL OF ABOVE CALCULATIONS =				$	3,600
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$	3,600.
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	3,600.
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$	3,600.

	Amount to be refunded:	$
	charged:	$

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. _18-0650_ in the amount of $ _3,600._ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. _18-0650_. A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Linda O. Palladino
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Linda O. Palladino
SIGNATURE   12/6/01

Linda O. Palladino
NAME

45,636
REGISTRATION NUMBER

Att. Docket No. REG 710-A-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of	:	Nicholas J. Papadopoulos, Samuel Davis, and George D. Yancopoulos
USSN	:	Not Yet Known
Filed	:	Filed Herewith
Int'l File No.	:	PCT/US00/14142
Int'l File Date	:	May 23, 2000
For	:	MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF

December 6, 2001

Commissioner for Patents
U.S. Patent and Trademark Office
Washington, D.C. 20231

Att:

PRELIMINARY AMENDMENT

Sir:

This paper is submitted in connection with the above-identified application. Prior to examination of the application on the merits, please amend the specification as follows:

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In the Claims:

Please replace Claim 9, starting on page 92, line 20, through page 93, line 10 with the following:

9. (Amended) An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4);

(b) the nucleotide sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the nucleotide sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the nucleotide sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the nucleotide sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14);

(g) the nucleotide sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16); and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c),

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(d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

Please replace Claim 22, starting on page 95, line 1, with the following:

22. (Amended) A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C (SEQ ID NOS: 15 and 16), which has been modified by acetylation or pegylation.

Please replace Claim 49, starting on page 98, line 24, through page 99, line 8, with the following:

49. (Amended) An fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4);

(b) the amino acid sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the amino acid sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the amino acid sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12)

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(f) the amino acid sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14); and

(g) the amino acid sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

In the Specification:

Please replace the paragraph starting on page 1, line 5, with the following:

The application claims priority of International Application No. PCT/US00/14142, filed May 23, 2000, which claims priority of U.S. Provisional Application No. 60/138,133, filed on June 8, 1999. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

Please replace the paragraph starting on page 11, line 15, through page 12, line 1, with the following:

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of (a) the nucleotide sequence set forth in Figure 13A-13D (SEQ ID NOS; 3 and 4);

(b) the nucleotide sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the nucleotide sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the nucleotide sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the nucleotide sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14);

(g) the nucleotide sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16); and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

Please replace the paragraph starting on page 13, line 6, with the following:

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C (SEQ ID NOS: 15 and 16), which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein

acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

Please replace the paragraph starting on page 15, line 19, with the following:

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4); (b) the amino acid sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6); (c) the amino acid sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8); (d) the amino acid sequence set forth in Figure 16A-16D (SEQ ID NOS 9 and 10); (e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12); (f) the amino acid sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14) and (g) the amino acid sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

Please replace the paragraph starting on page 19, line 11, with the following:

Figure 10A-10D (SEQ ID NOS: 1 and 2). Nucleic acid (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of Flt1(1-3)-Fc.

Please replace the paragraph starting on page 19, line 16, with the following:

Figure 13A-13D (SEQ ID NOS: 3 and 4). Nucleic acid (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

Please replace the paragraph starting on page 19, line 22, with the following:

Figure 14A-14 C (SEQ ID NOS: 5 and 6). Nucleic acid (SEQ ID NO: 5) and deduced amino acid sequence (SEQ ID NO: 6) of Mut2: Flt1(2-3$_{\Delta B}$)-Fc.

Please replace the paragraph starting on page 19, line 25, with the following:

Figure 15A-15C (SEQ ID NOS: 7 and 8). Nucleic acid (SEQ ID NO: 7) and deduced amino acid sequence (SEQ ID NO: 8) of Mut3: Flt1(2-3)-Fc.

Please replace the paragraph starting on page 20, line 1 with the following:

Figure 16A-16D (SEQ ID NOS. 9 and 10). Nucleic acid (SEQ ID NO: 9) and deduced amino acid sequence (SEQ ID NO: 10) of Mut4: Flt1(1-3$_{R->N}$)-Fc.

Please replace the paragraph starting on page 21, line 16, with the following:

Figure 21A-21C (SEQ ID NOS: 11 and 12). Nucleotide (SEQ ID NO: 11) and deduced amino acid sequence (SEQ ID NO: 12) of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

Please replace the paragraph starting on page 21, line 19, with the following:

Figure 22A-22C (SEQ ID NOS: 13 and 14). Nucleotide (SEQ ID NO: 13) and deduced amino acid sequence (SEQ ID NO: 14) of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Please replace the paragraph starting on page 22, line 1, with the following:

Figure 24A-24C (SEQ ID NOS: 15 and 16). Nucleotide (SEQ ID NO: 15) and deduced amino acid sequence (SEQ ID NO: 16) of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Please replace the paragraph starting on page 25, line 18, with the following:

Figure 36 (SEQ ID NO: 17). Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410. Please replace the paragraph starting on page 49, line 25, through page 51, line 12, with the following:

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus

sequences" for such heparin binding sites (see for example Hileman, et al.,
1998, Bioessays 20(2):156-67). In other cases, the known crystal
structure of a protein reveals a cluster of positively charged residues on
the surface of a protein, but the residues come from different regions of
the primary sequence and are only brought together when the protein
folds into its tertiary structure. Thus it is difficult to deduce whether an
isolated amino acid residue forms part of a cluster of basic residues on
the surface of the protein. However, if there is a cluster of positively
charged amino acid residues in the primary sequence, it is not
unreasonable to surmise that the residues are spatially close to one
another and might therefore be part of an extracellular matrix component
binding site. Flt1 receptor has been studied extensively and various
domains have been described (see for example Tanaka et al., 1997, Jpn. J.
Cancer Res 88:867-876). Referring to the nucleic acid and amino acid
sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) of this
application, one can identify the signal sequence for secretion which is
located at the beginning of the sequence and extends to the glycine coded
for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-
Lys (SEQ ID NO: 35), starting at nucleotide 79 of the nucleic acid
sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending
with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from
nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig
domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to
Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly,
encoded by nucleotides 997-1005, followed by the nucleotide sequence

encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to
Pro-Gly-Lys-stop).

Please replace the paragraph starting on page 51, line 14, through page
52, line 16, with the following:

A more detailed analysis of the Flt1 amino acid sequence reveals that
there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR
[SEQ ID NO: 36]) of Figure 10A-10D (SEQ ID NOS: 1 and 2), in which 6 out
of 10 amino acid residues are basic. This sequence is located in Flt1 Ig
domain 3 of the receptor (see Figure 11), which is not itself essential for
binding of VEGF ligand, but which confers a higher affinity binding to
ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain
2 reveals that in this region, there is very poor alignment between the two
Ig domains, and that there are about 10 additional amino acids in Ig domain
3. An analysis of the hydrophilicity profiles (MacVector computer
software) of these two domains clearly indicates the presence of a
hydrophilic region in the protein (Figure 12A-12B). These observations
raised the possibility that the actual three dimensional conformation of
Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig
domain 2. To test this hypothesis, the 10 additional amino acids were
deleted and the resulting protein was tested to see whether the deletion
would affect the pharmacokinetics favorably without seriously
compromising the affinity of the receptor for VEGF. This DNA construct,
which was constructed using standard molecular biology techniques (see

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e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-3$_{\Delta B}$)-Fc. The Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in Figure 10A-10D [SEQ ID NOS: 1 and 2]), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg (SEQ ID NO: 32) from Flt1 Ig domain 3.

Please replace the paragraph starting on page 52, line 18, with the following:

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3$_{\Delta B}$)-Fc is set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4).

Please replace the paragraph starting on page 53, line 4, with the following:

A second deletion mutant construct, designated Mut2: Flt1(2-3$_{\Delta B}$)-Fc, was derived from the Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D [SEQ ID

NOS: 1 and 2]); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-3$_{\Delta B}$)-Fc is set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6).

Please replace the paragraph starting on page 53, line 23, through page 54, line 4, with the following:

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-3$_{\Delta B}$)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8).

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Please replace the paragraph starting on page 54, line 9, with the following:

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D [SEQ ID NOS: 1 and 2]). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10).

Please replace the paragraph starting on page 60, line 4, with the following:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3' [SEQ ID NO: 18])

Please replace the paragraph starting on page 60, line 6, with the

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT [SEQ ID NO: 19])

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Please replace the paragraph starting on page 60, line 8, with the following:

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO: 20) (corresponding to amino acids 27-33 of Figure 21A-21C [SEQ ID NOS: 11 and 12]). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37)of Flt1 (corresponding to amino acids 123-126 of Figure 21A-21C [SEQ ID NOS: 11 and 12]) and continuing into VVLS (SEQ ID NO: 38) (corresponding to amino acids 127-130 of Figure 21A-21C [SEQ ID NOS: 11 and 12]) of Flk1.

Please replace the paragraph starting on page 60, line 20, with the following:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3' [SEQ ID NO: 21])

Please replace the paragraph starting on page 60, line 23, with the following:

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTTCATGGACCCTGAC AAATG-3' [SEQ ID NO: 22])

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Please replace the paragraph starting on page 61, line 1, with the following:

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO: 23) (corresponding to amino acids 223-228 of Figure 21A-21C [SEQ ID NOS: 11 and 12]), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 11 and 12).

Please replace the paragraph starting on page 61, line 10, with the following:

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested

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with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C [SEQ ID NOS: 11 and 12].

Please replace the paragraph starting on page 62, line 13, with the following:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3' [SEQ ID NO: 24])

Please replace the paragraph starting on page 62, line 15, with the following:

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT [SEQ ID NO: 25])

Please replace the paragraph starting on page 62, line 18, with the following:

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO: 20) (corresponding to amino acids 27-33 of Figure 22A-22C [SEQ ID

NOS: 13 and 14]). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37) of Flt1 (corresponding to amino acids 123-126 of Figure 22A-22C [SEQ ID NOS: 13 and 14]) and continuing into IQLL (SEQ ID NO: 26) of VEGFR3 (corresponding to amino acids 127-130 of Figure 22A-22C [SEQ ID NOS: 13 and 14]).

Please replace the paragraph starting on page 63, line 5, with the following:

5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA [SEQ ID NO: 27])

Please replace the paragraph starting on page 63, line 7, with the following:

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG [SEQ ID NO: 28])

Please replace the paragraph starting on page 63, line 16, with the following:

5':Flt1D2.VEGFR3D3.s
(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG [SEQ ID NO: 29])

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Please replace the paragraph starting on page 63, line 19, with the following:

3': VEGFR3D3/srf.as
(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT [SEQ ID NO: 30])

Please replace the paragraph starting on page 63, line 22, through page 64, line 4, with the following:

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO: 31) (corresponding to amino acids 221-226 of Figure 22A-22C [SEQ ID NOS: 13 and 14]), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C (SEQ ID NOS: 13 and 14).

Please replace the paragraph starting on page 64, line 6, with the following:

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were

19

combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14).

Please replace the paragraph starting on page 67, line 7, with the following:

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C [SEQ ID NOS: 15 and 16]) between Flt1d2-Flk1d3-FcΔC1(a) amino acids 26 and 27 of Figure 21A-21C (SEQ ID NOS: 11 and 12) (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 11 and 12). The SDT amino acid sequence is native to the Flt1 receptor and

20

was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

Please replace the paragraph starting on page 80, line 22, through page 81, line 6, with the following:

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36 (SEQ ID NO: 17).

Please replace the paragraph starting on page 81, line 20, through page 82, line 2, with the following:

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino

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acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation isobserved on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36 (SEQ ID NO: 17).

Please replace the paragraph starting on page 89, line 13, with the following:

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3$_{NAS}$)-Fc, Flt1(1-3$_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3 $_{NAS}$)-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR (SEQ ID NO: 32) is replaced by NASVNGSR (SEQ ID NO: 33), resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-3 $_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR (SEQ ID NO: 32) -> KNKCASVRRR [SEQ ID NO: 34]) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free

VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-$3_{R->C}$)-Fc, Flt1(1-3_{NAS})-Fc and Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

REMARKS

This Preliminary Amendment is made merely to insert the priority data and to add the sequence identifiers to the specification.

Applicants submit herewith as Exhibit A: Marked-Up Versions of pages 1, 11, 13, 15, 19, 20, 21, 22, 25, 49, 50, 51, 52, 53, 54, 60, 61, 62, 63, 64, 67, 80, 81, 82, 89, 90, 92, 93, 95, 98, and 99; Exhibit B: Sequence Listings in paper and computer readable forms.

I hereby state that the content of the paper readable and computer readable copy of the Sequence Listing submitted herewith and referred to herein in accordance with 37 C.F.R. § 1.821(g), contain no new subject matter.

Applicants direct the subject Sequence Listings submitted herewith be added to the specification.

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No fee is deemed necessary for filing this paper. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge any such fees required by this paper to Deposit Account No. 18-0650.

Respectfully submitted,

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MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED
PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND
USING THEREOF

This Application claims priority of International Application No.
PCT/US00/14142 filed, May 23, 2000, which

5   ~~The application~~ claims priority of U.S. Provisional Application No.

60/138,133, filed on June 8, 1999. Throughout this application various

publications are referenced. The disclosures of these publications in

their entireties are hereby incorporated by reference into this

application.

10

INTRODUCTION

The field of this invention is modified polypeptides with improved

pharmacokinetics. Specifically, the field of this invention relates to

15   Flt1 receptor polypeptides that have been modified in such a way as to

improve their pharmacokinetic profile. The field of this invention also

relates to methods of making and using the modified polypeptides

including but not limited to using the modified polypeptides to decrease

or inhibit plasma leakage and/or vascular permeability in a mammal.

20

BACKGROUND

The ability of polypeptide ligands to bind to cells and thereby elicit a

phenotypic response such as cell growth, survival, cell product

25   secretion, or differentiation is often mediated through transmembrane

receptors on the cells. The extracellular domain of such receptors (i.e.

that portion of the receptor that is displayed on the surface of the cell)

is generally the most distinctive portion of the molecule, as it provides

the protein with its ligand binding characteristic. Binding of a ligand

1

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain

5   3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

10   In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule

15   comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

(a) the nucleotide sequence set forth in Figure 13A-13D; *(SEQ ID NOS: 3 and 4)*

20   (b) the nucleotide sequence set forth in Figure 14A-14C; *(SEQ ID NOS: 5 and 6)*

(c) the nucleotide sequence set forth in Figure 15A-15C; *(SEQ ID NOS: 7 and 8)*

(d) the nucleotide sequence set forth in Figure 16A-16D; *(SEQ ID NOS: 9 and 10)*

(e) the nucleotide sequence set forth in Figure 21A-21C; *(SEQ ID NOS: 11 and 12)*

(f) the nucleotide sequence set forth in Figure 22A-22C; *(SEQ ID NOS: 13 and 14)*

(SEQ ID NOS: 15 and 16)

25   (g) the nucleotide sequence set forth in Figure 24A-24C; and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having

11

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

5

Additional embodiments include a fusion polypeptide encoded by the (SEQ ID NOS: 1 and 2) nucleic acid sequence set forth in Figure 10A-10D or Figure 24A-24C, (SEQ ID NOS: 15 and 16) which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar

10 excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

15 A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

20

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood

25 vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

13

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

Preferred embodiments include a fusion polypeptide wherein amino acid
5 sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the
10 amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an
15 embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an
20 amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D, *(SEQ ID NOS: 3 AND 4)* (b) the amino acid sequence set forth in Figure 14A-14C, *(SEQ ID NOS: 5 and 6)* (c) the amino acid sequence set forth in Figure *(SEQ ID NOS 9 and 10)* 15A-15C, *(SEQ ID NOS: 7 and 8)* (d) the amino acid sequence set forth in Figure 16A-16D; (e) *(SEQ ID NOS: 11 and 12)*
25 the amino acid sequence set forth in Figure 21A-21C, (f) the amino acid sequence set forth in Figure 22A-22C, *(SEQ ID NOS: 13 AND 14)* and (g) the amino acid sequence set forth in Figure 24A-24C. *(SEQ ID NOS: 15 and 16)*

unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The T_{max} for all of

5 the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the C_{max} was as follows: Unmodified Flt1(1-3)-Fc: 0.06µg/ml; 10 fold excess sample: - 0.7µg/ml, 20 fold excess sample - 2µg/ml, 40 fold excess sample - 4µg/ml, 60 fold excess sample - 2µg/ml, 100 fold excess sample - 1µg/ml.

10 (SEQ ID NOS: 1 and 2) → (SEQ ID NO: 1) (SEQ ID NO: 2)

Figure 10A-10D. Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

Figure 11. Schematic diagram of the structure of Flt1.

15 **Figure 12A and 12B.** Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

(SEQ ID NOS: 3 and 4)   (SEQ ID NO: 3)   (SEQ ID NO: 4)

Figure 13A-13D. Nucleic acid and deduced amino acid sequence of

20 Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

(SEQ ID NOS: 5 and 6) (SEQ ID NO: 5)   (SEQ ID NO: 6)

Figure 14A-14 C. Nucleic acid and deduced amino acid sequence of Mut2: Flt1(2-3$_{\Delta B}$)-Fc.

(SEQ ID NOS: 7 and 8) (SEQ ID NO: 7)   (SEQ ID NO: 8)

25 **Figure 15A-15C.** Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

(SEQ ID NOS: 9 and 10) (SEQ ID NO: 9)   (SEQ ID NO: 10)

Figure 16A-16D. Nucleic acid and deduced amino acid sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc.

Figure 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion

5   mutant Flt1(1-3)-Fc, and Flt1(1-3)$_{R->N}$ mutant proteins in a Biacore-based assay. At the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5

10   µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the

15   Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3$_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3$_{R->N}$)-Fc is somewhat less efficient at blocking binding

20

Figure 18. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the

25   Mut1: Flt1(1-3$_{\Delta B}$)-Fc protein binds more weakly still, and the Mut2: Flt1(2-3$_{\Delta B}$)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3$_{R->N}$)-Fc

20

glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

Figure 19. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc,

5 Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

10 **Figure 20.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-3$_{\Delta B}$)-Fc - 0.7μg/ml.

15 *(SEQ ID NOS: 11 and 12) (SEQ ID No: 11)*   *(SEQ ID No: 12)*
Figure 21A-21C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

(SEQ ID NOS: 13 and 14) (SEQ ID No: 13)   *(SEQ ID No: 14)*
Figure 22A-22C. Nucleotide and deduced amino acid sequence of the

20 modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Figure 23. Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the

25 ECM as compared to the Flt1(1-3)-Fc protein.

(SEQ ID NOS: 15 and 16)
(SEQ ID NO:15)
(SEQ ID NO: 16)

Figure 24A-24C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Figure 25A-25C. Phosphorylation assay. At a 1.5 molar excess of
5 either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient
Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor
stimulation by these three modified Flt1 receptors as compared to
control media challenge. In contrast, transient
Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this
10 molar excess, as compared with VEGF positive control challenge.
Similar results are seen in Figure 25B, where the modified Flt
receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure
25C, where the modified Flt1 receptors are in a 6-fold molar excess to
VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown
15 to be partially blocking VEGF165-induced stimulation of cell-surface
receptors.

Figure 26A-26B. Phosphorylation assay. Detection by Western blot
of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation
20 shows that cell-surface receptors are not phosphorylated by challenge
samples which have VEGF165 preincubated with 1 and 2 fold molar
excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either
transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or
transient VEGFR1R2-FcΔC1(a). At all modified Flt1 receptor
25 concentrations tested there is complete binding of VEGF165 ligand

22

Figure 33, Figure 34 and Figure 35. Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering

5 detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165.

10 MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak

15 position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

(SEQ ID NO: 17)

Figure 36. Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were

20 determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two

25 cysteines in another Fc chain. However, it can not be determined

(d.) Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic

5 profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc

protein. Balb/c mice (23-28g) were injected subcutaneously with

4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess

samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20

and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar

10 excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days

and 3 days after injection. The sera were assayed in an ELISA-based

assay designed to detect Flt1(1-3)-Fc (described *supra*). Figure 9

details the results of this study. The Tmax for all of the Flt1(1-3)-Fc

proteins tested was at the 6 hour time point but the Cmax was as

15 follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold molar excess

sample: - 0.7μg/ml, 20 fold molar excess sample - 2μg/ml, 40 fold

molar excess sample - 4μg/ml, 60 fold molar excess sample - 2μg/ml,

100 fold molar excess sample - 1μg/ml. This results demonstrates

that acetylation or pegylation of Flt1(1-3)-Fc significantly improves

20 its pharmacokinetic profile.

Example 11: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

25 Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI

49

below 6, has much better pharmacokinetics than the highly positive

unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference

in pharmacokinetics could be attributed to the net charge of the

protein, which made it stick to negatively charged extracellular matrix

5　components, or whether there were perhaps specific locations on the

surface of the Flt1(1-3)-Fc protein that constituted specific binding

sites for extracellular matrix components.　For example, many proteins

are known to have heparin binding sites, often consisting of a cluster of

basic residues.　Sometimes these residues are found in a cluster on the

10　primary sequence of the protein; some of the literature has identified

"consensus sequences" for such heparin binding sites (see for example

Hileman, et al., 1998, Bioessays 20(2):156-67).　In other cases, the

known crystal structure of a protein reveals a cluster of positively

charged residues on the surface of a protein, but the residues come

15　from different regions of the primary sequence and are only brought

together when the protein folds into its tertiary structure.　Thus it is

difficult to deduce whether an isolated amino acid residue forms part

of a cluster of basic residues on the surface of the protein.　However, if

there is a cluster of positively charged amino acid residues in the

20　primary sequence, it is not unreasonable to surmise that the residues

are spatially close to one another and might therefore be part of an

extracellular matrix component binding site.　Flt1 receptor has been

studied extensively and various domains have been described (see for

example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876).　Referring

25　to the nucleic acid and amino acid sequence set forth in Figure 10A-10D

(SEQ ID NOS: 1 and 2)

50

of this application, one can identify the signal sequence for secretion

which is located at the beginning of the sequence and extends to the

glycine coded for by nucleotides 76-78. The mature protein begins with

(SEQ ID NO. 35)

Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid

5   sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending

with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from

nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1

Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val

to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly,

10   encoded by nucleotides 997-1005, followed by the nucleotide sequence

encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys

to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that

[SEQ ID NO 36]

15   there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR)

(SEQ ID NOS: 1 and 2)

of Figure 10A-10D, in which 6 out of 10 amino acid residues are basic.

This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure

11), which is not itself essential for binding of VEGF ligand, but which

confers a higher affinity binding to ligand. An alignment of the

20   sequence of Ig domain 3 with that of Ig domain 2 reveals that in this

region, there is very poor alignment between the two Ig domains, and

that there are about 10 additional amino acids in Ig domain 3. An

analysis of the hydrophilicity profiles (MacVector computer software)

of these two domains clearly indicates the presence of a hydrophilic

25   region in the protein (Figure 12A-12B). These observations raised the

possibility that the actual three dimensional conformation of Flt1 Ig

domain 3 allowed for some type of protrusion that is not in Flt1 Ig

domain 2. To test this hypothesis, the 10 additional amino acids were

deleted and the resulting protein was tested to see whether the

5   deletion would affect the pharmacokinetics favorably without seriously

compromising the affinity of the receptor for VEGF. This DNA

construct, which was constructed using standard molecular biology

techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook,

et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular

10   Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience,

NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc.,

Cambridge, MA), is referred to as Mut1: Flt1$(1-3_{\Delta B})$-Fc. The Mut1:

Flt1$(1-3_{\Delta B})$-Fc construct was derived from Flt1(1-3)-Fc by deletion of
(SEQ ID NOS: 1 and 2)

nucleotides 814-843 (set forth in Figure 10A-10D), which deletes the

15   highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-
(SEQ ID NO: 32)

Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA

sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied

20   Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1$(1-3_{\Delta B})$-
(SEQ ID NOS: 3 and 4)

Fc is set forth in Figure 13A-13D.

Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3 $_{\Delta B}$)-Fc.

A second deletion mutant construct, designated Mut2: Flt1(2-3$_{\Delta B}$)-Fc,

5 was derived from the Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct by deletion of Flt1 *(SEQ ID NOS: 1 and 2)* Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D); for

convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA.

This introduced a restriction site (BspE1) without altering the

associated amino acid sequence, Ser-Gly. This DNA construct, which

10 was constructed using standard molecular biology techniques (see e.g.,

Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring

Harbor Laboratory), Current Protocols in Molecular Biology (Eds.

Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the

mammalian expression vector pMT21 (Genetics Institute, Inc.,

15 Cambridge, MA), was also sequence-verified using an ABI 373A DNA

sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied

Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-3$_{\Delta B}$)- *(SEQ ID NOS: 5 and 6)*

Fc is set forth in Figure 14A-14C.

Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was

constructed the same way as the Mut2: Flt1(2-3$_{\Delta B}$)-Fc construct,

25 except that Flt1 Ig domain 3 was left intact (the basic region amino

acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C. (SEQ ID NOS: 7 and 8)

5

Example 14: Construction of Flt(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3 _{R->N})-Fc.

10   A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). [SEQ ID NOS: 1 and 2] The resulting amino acid sequence is therefore changed from

15   Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D. (SEQ ID NOS: 9 and 10)

20   **Example 15: Characterization of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and Mut4: Flt1(1-3$_{R->N}$)-Fc mutants.**

(a.) Binding to extracellular matrix components

25   To determine whether the three modified proteins were more or less

54

single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification

primers were as follows:

[SEQ ID NO: 18]

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

5

[SEQ ID NO: 19]

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3')

The 5' amplification primer encodes a BspE1 restriction enzyme site

upstream of Ig domain 2 of Flt1, defined by the amino acid sequence

(SEQ ID NO: 20)   [SEQ ID NOS: 11 and 12]

10   GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C). The

3' primer encodes the reverse complement of the 3' end of Flt1 Ig

domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with

(SEQ ID NO: 37)

the fusion point defined as TIID of Flt1 (corresponding to amino acids

[SEQ ID NOS: 11 and 12]   (SEQ ID NO: 38)

123-126 of Figure 21A-21C) and continuing into VVLS (corresponding

[SEQ ID NOS. 11 and 12]

15   to amino acids 127-130 of Figure 21A-21C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as

follows:

20   5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG

[SEQ ID NO: 21]

G-3')

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC

[SEQ ID NO: 22]

AAATG-3')

25

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused

directly to the beginning of Flk1 Ig domain 3, as described above. The

3' amplification primer encodes the end of Flk1 Ig domain 3, defined by

(SEQ ID NO. 23)

the amino acids VRVHEK (corresponding to amino acids 223-228 of

[SEQ ID NOS: 11 and 12]

5   Figure 21A-21C), followed by a bridging sequence that includes a

recognition sequence for the restriction enzyme Srf1, and encodes the

amino acids GPG. The bridging sequence corresponds to amino acids

(SEQ ID NOS: 11 and 12)

229-231 of Figure 21A-21C.

10   After a round of PCR amplification to produce the individual domains,

the products were combined in a tube and subjected to a further round

of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described

supra) to produce the fusion product. This PCR product was

subsequently digested with the restriction enzymes BspEI and SmaI and

15   the resulting 614bp fragment was subcloned into the BspEI to SrfI

restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid

pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3

gene fusion insert was verified by standard sequence analysis. This

plasmid was then digested with the restriction enzymes EcoRI and SrfI

20   and the resulting 702bp fragment was transferred into the EcoRI to SrfI

restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the

plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino

acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set

(SEQ ID NOS: 11 and 12)

forth in Figure 21A-21C.

25

(b)   Construction of the expression plasmid pFlt1D2VEGFR3D3FcΔC1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin

5   resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1

receptor.   This plasmid was used to produce a DNA fragment containing

Ig domain 2 of Flt1 by PCR.   RNA from the cell line HEL921.7 was used

to produce Ig domain 3 of Flk1, using standard RT-PCR methodology.   A

further round of PCR amplification was used to achieve fusion of the

10   two Ig domains into a single fused fragment.   For Ig domain 2 of Flt1,

the 5' and 3' amplification primers were as follows:

[SEQ ID NO. 24]

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

15   3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA
      [SEQ ID NO: 25]
TTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream
                                                                    (SEQ ID NO. 20)
of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM
                                                          [SEQ ID NOS: 13 AND 14]
20   (corresponding to amino acids 27-33 of Figure 22A-22C).   The 3'

amplification primer encodes the reverse complement of the end of Flt1

Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with
                                        SEQ ID NO: 37
the fusion point defined as TIID of Flt1 (corresponding to amino acids
                      [SEQ ID NOS: 13 and 14]   (SEQ ID NO. 26)
123-126 of Figure 22A-22C) and continuing into IQLL of VEGFR3
                                                        [SEQ ID NOS: 13 and 14]
25   (corresponding to amino acids 127-130 of Figure 22A-22C).

62

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

[SEQ ID NO.27]

5   5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

[SEQ ID NO.28]

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3.
10   The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

15

5':Flt1D2.VEGFR3D3.s   [SEQ ID NO: 29]

(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as   [SEQ ID NO: 30]

20   (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of
(SEQ ID NO: 31)
25   VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding

[SEQ ID NOS: 13 and 14]

to amino acids 221-226 of Figure 22A-22C), followed by a bridging

sequence that includes a recognition sequence for Srf1, and encodes the

amino acids GPG. The bridging sequence corresponds to amino acids

(SEQ ID NOS: 13 and 14)

227-229 of Figure 22A-22C.

5

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain

3) of PCR to produce the individual Ig domains, the PCR products were

combined in a tube and subjected to a further round of PCR

amplification with the amplification primers bsp/flt1D2 and

10   VEGFR3D3/srf.as described *supra*, to produce the fusion product. This

PCR product was subsequently digested with the restriction enzymes

BspEI and SmaI and the resulting 625bp fragment was subcloned into

the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc

(described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc.

15   The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified

by standard sequence analysis. This plasmid was then digested with

the restriction enzymes EcoRI and SrfI and the resulting 693bp

fragment was subcloned into the EcoRI to SrfI restriction sites of the

plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated

20   pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid

sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set

(SEQ ID NOS: 13 and 14)

forth in Figure 22A-22C.

cells.   The supernatant was decanted into sterile 1L bottles and

purification of the expressed protein was performed as described *infra.*

Example 20:   Construction pVEGFR1R2-FcΔC1(a) expression

5   **vector**

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by

insertion of DNA encoding amino acids SDT (corresponding to amino

[SEQ ID NOS: 15 and 16]

acids 27-29 of Figure 24A-24C) between Flt1d2-Flk1d3-FcΔC1(a)

(SEQ ID NOS: 11 and 12)

10   amino acids 26 and 27 of Figure 21A-21C (GG) and removal of DNA

encoding amino acids GPG corresponding to amino acids 229-231 of

21A-21C (SEQ ID NOS: 11 and 12)

Figure.   The SDT amino acid sequence is native to the Flt1 receptor and

was added back in to decrease the likelihood of heterogeneous N-

terminal processing.   The GPG (bridging sequence) was removed so that

15   the Flt1 and Flk1 Ig domains were fused directly to one another.   The

complete DNA and deduced amino acid sequences of the

pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C. (SEQ ID NOS: 15 and 16)

Example 21:   Cell Culture Process Used to Produce Modified

20   **Flt1   Receptors**

(a) Cell Culture Process Used to Produce

Flt1D2.Flk1D3.FcΔC1(a)

25   The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the

(Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was

5 calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390

10 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165.

15 Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

20 **Example 28:   Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)**

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic

25 fragments were analyzed and identified by HPLC coupled with mass

80

spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify

5 fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36. (SEQ ID NO. 17)

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide

10 bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined

15 whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

20 There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is

81

observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36. (SEQ ID NO: 17)

Example 29: Pharmacokinetic Analysis of Modified Flt

5 ### Receptors

(a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)

10   Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24hrs, 2 days, 3 days and 6 days after injection. The sera were assayed

15   in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results

20   of this experiments are shown in Figure 37. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max}

Example 33: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and

5 tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

10 ## Example 34: VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The

15 modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3$_{NAS}$)-Fc, Flt1(1-3$_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3$_{NAS}$)-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic

   (SEQ ID NO: 30) *(SEQ ID NO. 33)*

20 amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-3 $_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same

   (SEQ ID NO: 33)

25 basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR ->

[SEQ ID NO. 34]

KNKCASVRR$\hat{R}$) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was

5   then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3) (A40) and then by Flt1(1-3$_{R->C}$)-Fc, Flt1(1-3$_{NAS}$)-Fc and

10   Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

5.   The isolated nucleic acid molecule of claim 1, wherein the

nucleotide sequence encoding Ig domain 2 of the extracellular domain of

the first VEGF receptor is upstream of the nucleotide sequence

encoding Ig domain 3 of the extracellular domain of the second VEGF

5   receptor.

6.   The isolated nucleic acid molecule of claim 1, wherein the

nucleotide sequence encoding Ig domain 2 of the extracellular domain of

the first VEGF receptor is downstream of the nucleotide sequence

10   encoding Ig domain 3 of the extracellular domain of the second VEGF

receptor.

7.   The isolated nucleic acid molecule of claim 1, wherein the

multimerizing component comprises an immunoglobulin domain.

15

8.   The isolated nucleic acid molecule of claim 1, wherein the

immunoglobulin domain is selected from the group consisting of the Fc

domain of IgG, the heavy chain of IgG, and the light chain of IgG.

(AMENDED)

20   9. ^An isolated nucleic acid molecule comprising a nucleotide sequence

encoding a modified Flt1 receptor fusion polypeptide, wherein the

coding region of the nucleic acid molecule consists of a nucleotide

sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 13A-13D; (SEQ ID NOS. 3 and 4)

25   (b) the nucleotide sequence set forth in Figure 14A-14C; (SEQ ID NOS. 5 and 6

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(c) the nucleotide sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 and 8)

(d) the nucleotide sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 and 10)

(e) the nucleotide sequence set forth in Figure 21A-21C; (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C; (SEQ ID NOS: 13 and 14)

(SEQ ID NOS: 15 and 16)

5   (g) the nucleotide sequence set forth in Figure 24A-24C; and

(h) a nucleotide sequence which, as a result of the degeneracy of

the genetic code, differs from the nucleotide sequence of (a), (b), (c),

(d), (e), (f), or (g) and which encodes a fusion polypeptide molecule

having the biological activity of the modified Flt1 receptor fusion

10   polypeptide.

10.   A fusion polypeptide encoded by the isolated nucleic acid molecule

of claim 1, 2, 3, 4 or 9.

15   11.   A composition capable of binding a VEGF molecule to form a

nonfunctional complex comprising a multimer of the fusion polypeptide

of claim 10.

12.   The composition of claim 11, wherein the multimer is a dimer.

20

13.   The composition of claim 12 and a carrier.

14.   A vector which comprises the nucleic acid molecule of claim 1, 2,

3, 4 or 9.

25

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(AmenOED)

22. A fusion polypeptide encoded by the nucleic acid sequence set forth
(SEQ ID NOS. 1 and 2) (SEQ ID NOS. 15 AND 16)
in Figure 10A-10D or Figure 24A-24C, which has been modified by
acetylation or pegylation.

5 23. The fusion polypeptide of claim 22 wherein the modification is
acetylation.

24. The fusion polypeptide of claim 22 wherein the modification is
pegylation.

10

25. The fusion polypeptide of claim 23 wherein the acetylation is
accomplished with at least about a 100 fold molar excess of
acetylation reagent.

15 26. The fusion polypeptide of claim 23 wherein acetylation is
accomplished with a molar excess of acetylation reagent ranging from
at least about a 10 fold molar excess to about a 100 fold molar excess.

27. The fusion polypeptide of claim 24 wherein the pegylation is 10K
20 or 20K PEG.

28. A method of decreasing or inhibiting plasma leakage in a mammal
comprising administering to the mammal fusion polypeptide of claim
10.

25

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43. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flk1.

44. The fusion polypeptide of claim 41 wherein the second VEGF

5   receptor is Flt4.

45. The fusion polypeptide claim 41, wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular

10   domain of the second VEGF receptor.

46. The fusion polypeptide of claim 41, wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of

15   the extracellular domain of the second VEGF receptor.

47. The fusion polypeptide of claim 41, wherein the multimerizing component comprises an immunoglobulin domain.

20   48. The fusion polypeptide of claim 41, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

(Amended)
49. ^An fusion polypeptide comprising an amino acid sequence of a

25   modified Flt1 receptor, wherein the amino acid sequence selected from

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the group consisting of:

(a) the amino acid sequence set forth in Figure 13A-13D; (SEQ ID NOS: 3 AND 4)

(b) the amino acid sequence set forth in Figure 14A-14C; (SEQ ID NOS: 5 AND 6)

(c) the amino acid sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 and 8)

5   (d) the amino acid sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 AND 10)

(e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12);

(f) the amino acid sequence set forth in Figure 22A-22C; and (SEQ ID NOS: 13 AND 14)

(g) the amino acid sequence set forth in Figure 24A-24C. (SEQ ID NOS: 15 AND 16)

10   50.   A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 41, 42, 43, 44 or 49.

51.   A method of inhibiting VEGF receptor ligand activity in a mammal 15 comprising administering to the mammal an effective amount of the fusion polypeptide of claim 41, 42, 43, 44 or 49.

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MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED
PHARMACOKINETIC PROPERTIES

5   The application claims priority of U.S. Provisional Application No.
60/138,133, filed on June 8, 1999.   Throughout this application various
publications are referenced.   The disclosures of these publications in
their entireties are hereby incorporated by reference into this
application.

10

INTRODUCTION

The field of this invention is modified polypeptides with improved
pharmacokinetics.   Specifically, the field of this invention relates to
15   Flt1 receptor polypeptides that have been modified in such a way as to
improve their pharmacokinetic profile.   The field of this invention also
relates to methods of making and using the modified polypeptides
including but not limited to using the modified polypeptides to decrease
or inhibit plasma leakage and/or vascular permeability in a mammal.

20

BACKGROUND

The ability of polypeptide ligands to bind to cells and thereby elicit a
phenotypic response such as cell growth, survival, cell product
25   secretion, or differentiation is often mediated through transmembrane
receptors on the cells.   The extracellular domain of such receptors (i.e.
that portion of the receptor that is displayed on the surface of the cell)
is generally the most distinctive portion of the molecule, as it provides
the protein with its ligand binding characteristic.   Binding of a ligand

to the extracellular domain generally results in signal transduction which transmits a biological signal to intracellular targets. Often, this signal transduction acts via a catalytic intracellular domain. The particular array of sequence motifs of this catalytic intracellular

5   domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol. 11:5068-5078; Fantl, et al., 1992, Cell 69:413-413). Examples of receptors that transduce signals via catalytic intracellular domains include the receptor tyrosine kinases (RTKs) such as the Trk family of receptors which are generally

10   limited to cells of the nervous system, the cytokine family of receptors including the tripartate CNTF receptor complex (Stahl & Yancopoulos, 1994, J. Neurobio. 25:1454-1466) which is also generally limited to the cells of the nervous system, G-protein coupled receptors such as the β_2-adrenergic receptor found on, for instance, cardiac muscle cells, and

15   the multimeric IgE high affinity receptor FcεRI which is localized, for the most part, on mast cells and basophils (Sutton & Gould, 1993, Nature 366:421-428).

All receptors identified so far appear to undergo dimerization,

20   multimerization, or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich & Schlessinger, 1990, Cell 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic

25   function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al., 1988, Science, 240:1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of epidermal growth

factor (EGF), the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem. 259:14631-14636). In the case of the FcεRI receptor, the ligand, IgE, exists bound to FcεRI in a monomeric fashion and only becomes activated when antigen binds to the IgE/FcεRI complex and cross-links

5   adjacent IgE molecules (Sutton & Gould, 1993, Nature 366:421-428).

Often, the tissue distribution of a particular receptor within higher organisms provides insight into the biological function of the receptor. The RTKs for some growth and differentiation factors, such as

10   fibroblast growth factor (FGF), are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol. 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting

15   of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which bind the Trk RTK family receptors, promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S.E. Loughlin & J.H. Fallon, eds., pp. 257-284, San

20   Diego, CA, Academic Press). FcεRI is localized to a very limited number of types of cells such as mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d.

25   Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

Many studies have demonstrated that the extracellular domain of a receptor provides the specific ligand binding characteristic. Furthermore, the cellular environment in which a receptor is expressed may influence the biological response exhibited upon binding of a ligand

5   to the receptor. For example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds to that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413).

10

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF has been purified from conditioned growth media of rat glioma cells [Conn et al., (1990), Proc.

15   Natl. Acad. Sci. U.S.A., 87. pp 2628-2632]; and conditioned growth media of bovine pituitary follicle stellate cells [Ferrara and Henzel, (1989), Biochem. Biophys. Res. Comm., 161, pp. 851-858; Gozpadorowicz et al., (1989), Proc. Natl. Acad. Sci. U.S.A., 86, pp. 7311-7315] and conditioned growth medium from human U937 cells [Connolly, D. T. et al. (1989),

20   Science, 246, pp. 1309-1312]. VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. VEGF has some structural similarities to platelet derived growth factor (PDGF), which is a mitogen for connective tissue cells but not mitogenic for vascular endothelial cells

25   from large vessels.

The membrane-bound tyrosine kinase receptor, known as Flt, was shown to be a VEGF receptor [DeVries, C. et al., (1992), Science, 255, pp.989-

991]. The Flt receptor specifically binds VEGF which induces
mitogenesis. Another form of the VEGF receptor, designated KDR, is
also known to bind VEGF and induce mitogenesis. The partial cDNA
sequence and nearly full length protein sequence of KDR is known as
5   well [Terman, B. I. et al., (1991) Oncogene 6, pp. 1677-1683; Terman, B.
I. et al., (1992) Biochem. Biophys. Res. Comm. 187, pp. 1579-1586].

Persistent angiogenesis may cause or exacerbate certain diseases such
as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas,
10   diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF
activity would be useful as a treatment for such diseases and other
VEGF-induced pathological angiogenesis and vascular permeability
conditions, such as tumor vascularization.   The present invention
relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.
15

Plasma leakage, a key component of inflammation, occurs in a distinct
subset of microvessels.   In particular, in most organs plasma leakage
occurs specifically in the venules.   Unlike arterioles and capillaries,
venules become leaky in response to numerous inflammatory mediators
20   including histamine, bradykinin, and serotonin. One characteristic of
inflammation is the plasma leakage that results from intercellular
gaps that form in the endothelium of venules.   Most experimental
models of inflammation indicate that these intercellular gaps occur
between the endothelial cells of postcapillary and collecting venules
25   (Baluk, P., et al., Am. J. Pathol. 1998 152:1463-76).   It has been shown
that certain lectins may be used to reveal features of focal sites of
plasma leakage, endothelial gaps, and finger-like processes at
endothelial cell borders in inflamed venules (Thurston, G., et al., Am. J.

Physiol, 1996, 271: H2547-62). In particular, plant lectins have been
used to visualize morphological changes at endothelial cell borders in
inflamed venules of, for example, the rat trachea. Lectins, such as
conconavalin A and ricin, that bind focally to inflamed venules reveal

5   regions of the subendothelial vessel wall exposed by gaps that
correspond to sites of plasma leakage (Thurston, G., et al., Am J
Physiol, 1996, 271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory

10   diseases, for example, are associated with microvascular remodeling,
including angiogenesis and microvessel enlargement. Microvessels can
also remodel by acquiring abnormal phenotypic properties. In a murine
model of chronic airway inflammation, airway capillaries acquire
properties of venules, including widened vessel diameter, increased

15   immunoreactivity for von Willebrand factor, and increased
immunoreactivity for P-selectin. In addition, these remodeled vessels
leak in response to inflammatory mediators, whereas vessels in the
same position in the airways of normal mice do not.

20   Certain substances have been shown to decrease or inhibit vascular
permeability and/or plasma leakage. For example, mystixins are
synthetic polypeptides that have been reported to inhibit plasma
leakage without blocking endothelial gap formation (Baluk, P., et al., J.
Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic

25   receptor agonist formoterol reduces microvascular leakage by
inhibiting endothelial gap formation (Baluk, P. and McDonald, D.M., Am. J.
Physiol., 1994, 266:L461-8).

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors thought to be largely specific for vascular endothelial cells.   Targeted gene inactivation studies in mice have shown that VEGF is necessary for the early stages

5   of vascular development and that Ang-1 is required for later stages of vascular remodeling.

US Patent No. 6,011,003, issued January 4, 2000, in the name of Metris Therapeutics Limited, discloses an altered, soluble form of FLT

10   polypeptide being capable of binding to VEGF and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains.

US Patent No. 5,712,380, issued January 27, 1998 and assigned to Merck

15   & Co., discloses vascular endothelial cell growth factor (VEGF) inhibitors that are naturally occurring or recombinantly engineered soluble forms with or without a C-terminal transmembrane region of the receptor for VEGF.

20   Also assigned to Merck & Co. is PCT Publication No. WO 98/13071, published April 2, 1998, which discloses gene therapy methodology for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding a soluble receptor protein which binds to VEGF.

25

PCT Publication No. WO 97/44453, published November 27, 1997, in the name of Genentech, Inc., discloses novel chimeric VEGF receptor proteins comprising amino acid sequences derived from the vascular

endothelial growth factor (VEGF) receptors Flt1 and KDR, including the murine homologue to the human KDR receptor FLK1, wherein said chimeric VEGF receptor proteins bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity thereof.

5

PCT Publication No. WO 97/13787, published April 17, 1997, in the name of Toa Gosei Co., LTD., discloses a low molecular weight VEGF inhibitor usable in the treatment of diseases accompanied by neovascularization such as solid tumors. A polypeptide containing the

10 first immunoglobulin-like domain and the second immunoglobulin-like domain in the extracellular region of a VEGF receptor FLT but not containing the sixth immunoglobulin-like domain and the seventh immunoglobulin-like domain thereof shows a VEGF inhibitory activity.

15 Sharifi, J. et al., 1998, The Quarterly Jour. of Nucl. Med. 42:242-249, disclose that because monoclonal antibodies (MAbs) are basic, positively charged proteins, and mammalian cells are negatively charged, the electrostatic interactions between the two can create higher levels of background binding resulting in low tumor to normal

20 organ ratios. To overcome this effect, the investigators attempted to improve MAb clearance by using various methods such as secondary agents as well as chemical and charge modifications of the MAb itself.

Jensen-Pippo, et al., 1996, Pharmaceutical Research 13:102-107,

25 disclose that pegylation of a therapeutic protein, recombinant human granulocyte colony stimulating factor (PEG-G-CSF), results in an increase in stability and in retention of *in vivo* bioactivity when administered by the intraduodenal route.

Tsutsumi, *et al.*, 1997, Thromb Haemost. 77:168-73, disclose experiments wherein the *in vivo* thrombopoietic activity of polyethylene glycol-modified interleukin-6 (MPEG-IL-6), in which 54%

5   of the 14 lysine amino groups of IL-6 were coupled with PEG, was compared to that of native IL-6.

Yang, *et al.*, 1995, Cancer 76:687-94, disclose that conjugation of polyethylene glycol to recombinant human interleukin-2 (IL-2) results

10   in a compound, polyethylene glycol-modified IL-2 (PEG-IL-2) that retains the in vitro and in vivo activity of IL-2, but exhibits a markedly prolonged circulating half-life.

R. Duncan and F. Spreafico, Clin. Pharmacokinet. 27: 290-306, 296

15   (1994) review efforts to improve the plasma half-life of asparaginase by conjugating polyethylene glycol.

PCT International Publication No. WO 99/03996 published January 28, 1999 in the name of Regeneron Pharmaceuticals, Inc. and The Regents of

20   The University of California describes modified human noggin polypeptides having deletions of regions of basic amino acids. The modified human noggin polypeptides are described as retaining biological activity while having reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to the

25   unmodified human noggin.

SUMMARY OF THE INVENTION

The present invention is directed to VEGF antagonists with improved pharmacokinetic properties. A preferred embodiment is an isolated

5   nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion

10 polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

15

In a further embodiment, the isolated nucleic acid of the first VEGF receptor is Flt1.

In a further embodiment, the isolated nucleic acid of the second VEGF

20 receptor is Flk1.

In yet another embodiment, the isolated nucleic acid of the second VEGF receptor is Flt4.

25 In another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain

5   3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

10   In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule

15   comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

(a) the nucleotide sequence set forth in Figure 13A-13D;

20   (b) the nucleotide sequence set forth in Figure 14A-14C;

(c) the nucleotide sequence set forth in Figure 15A-15C;

(d) the nucleotide sequence set forth in Figure 16A-16D;

(e) the nucleotide sequence set forth in Figure 21A-21C;

(f) the nucleotide sequence set forth in Figure 22A-22C;

25   (g) the nucleotide sequence set forth in Figure 24A-24C; and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having

11

the biological activity of the modified Flt1 receptor fusion polypeptide.

In a further embodiment of the invention, a fusion polypeptide is encoded by the isolated nucleic acid molecules described above.

5

A preferred embodiment is a composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide.

10   Also preferred is a composition wherein the multimer is a dimer.

In yet another embodiment, the composition is in a carrier.

Another embodiment is a vector which comprises the nucleic acid

15   molecules described above, including an expression vector comprising a the nucleic acid molecules described wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Other included embodiments are a host-vector system for the

20   production of a fusion polypeptide which comprises the expression vector, in a suitable host cell; the host-vector system wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell; the host-vector system wherein the suitable host cell is E. Coli; the host-vector system wherein the suitable host cell is a

25   COS cell; the host-vector system wherein the suitable host cell is a CHO cell.

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

5

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D or Figure 24A-24C, which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar

10   excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

15   A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

20

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood

25   vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

13

Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

5   Preferred embodiments of these methods are wherein the mammal is a human.

Further embodiments of the methods of the invention include attenuation or prevention of tumor growth in a human; attenuation or
10   prevention of edema in a human, especially wherein the edema is brain edema; attenuation or prevention of ascites formation in a human, especially wherein the ascites is ovarian cancer-associated ascites.

Preferred embodiments of the invention include a fusion polypeptide
15   capable of binding a VEGF polypeptide comprising (a) a VEGF receptor component operatively linked to (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a
20   first VEGF receptor and the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

In a further embodiment of the fusion polypeptide, the first VEGF receptor is Flt1.

25

In yet a further embodiment of the fusion polypeptide, the second VEGF receptor is Flk1.

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

5   Preferred embodiments include a fusion polypeptide wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the

10   amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an

15   embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an

20   amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D; (b) the amino acid sequence set forth in Figure 14A-14C; (c) the amino acid sequence set forth in Figure 15A-15C; (d) the amino acid sequence set forth in Figure 16A-16D; (e)

25   the amino acid sequence set forth in Figure 21A-21C; (f) the amino acid sequence set forth in Figure 22A-22C; and (g) the amino acid sequence set forth in Figure 24A-24C.

Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.

5 An alternative preferred embodiment is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

BRIEF DESCRIPTION OF THE FIGURES

10

Figure 1. IEF gel analysis of unmodified and acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas acetylated Flt1(1-3)-Fc is able to enter the gel and equilibrate at pI 5.2.

15

Figure 2. Binding of unmodified Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc proteins binds extensive to extracellular matrix components in Matrigel®, whereas acetylated Flt1(1-3)-Fc does not bind.

20

Figure 3. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc in a Biacore-based assay. Acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete

25 with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appears to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding.

16

However, washing the bound samples with 0.5M NaCl (columns 7-8) results in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein is exhibiting non-specific binding to the chip that can be eliminated by the salt wash.

5

Figure 4. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc to VEGF in an ELISA-based assay. Both pegylated and acetylated Flt1(1-3)-Fc proteins bind to VEGF with affinities approaching that of unmodified Flt1(1-3)-Fc.

10

Figure 5. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2,

15 4, 6, 24 hours, 2 days, and 3 days after injection of protein and the sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. The T_{max} for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The C_{max} for the different proteins was as follows: Unmodified: 0.06 µg/ml - 0.15 µg/ml;

20 acetylated: 1.5 µg/ml - 4.0 µg/ml; and pegylated: approximately 5 µg/ml.

Figure 6A-6B. IEF gel analysis of unmodified and step-acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to

25 enter the gel due to its >9.3 pI, whereas most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation.

Figure 7. Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. As with the irrelevant control protein, rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20

5   and 30 fold excess samples) does not exhibit any binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. The 10 fold excess sample shows reduced binding, but the degree of acetylation is not enough to completely block binding to extracellular matrix components.

10

Figure 8. Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc in a Biacore-based assay. At a sub-stoichiometric ratio (0.5 µg/ml of either unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either

15   unmodified or step-acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometric ratio, the both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely saturate

20   the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

25   **Figure 9.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for

unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The T_{max} for all of

5   the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the C_{max} was as follows: Unmodified Flt1(1-3)-Fc: 0.06µg/ml; 10 fold excess sample: - 0.7µg/ml, 20 fold excess sample - 2µg/ml, 40 fold excess sample - 4µg/ml, 60 fold excess sample - 2µg/ml, 100 fold excess sample - 1µg/ml.

10

Figure 10A-10D. Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

Figure 11. Schematic diagram of the structure of Flt1.

15

Figure 12A and 12B. Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

Figure 13A-13D. Nucleic acid and deduced amino acid sequence of
20   Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

Figure 14A-14 C. Nucleic acid and deduced amino acid sequence of Mut2: Flt1(2-3$_{\Delta B}$)-Fc.

25   **Figure 15A-15C.** Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

Figure 16A-16D. Nucleic acid and deduced amino acid sequence of Mut4: Flt1($1-3_{R->N}$)-Fc.

Figure 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion

5   mutant Flt1(1-3)-Fc, and Flt1(1-3)$_{R->N}$ mutant proteins in a Biacore-

based assay. At the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc

of unmodified, acetylated or genetically modified samples vs. 01.

µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding

of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5

10   µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc

proteins, the stoichiometric ratio approximates 1:1 and there is an

increased ability to block VEGF binding to the Biacore chip. At 1.0

µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc

proteins, which is approximately a 10:1 stoichiometric ratio, the

15   Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore

chip, but they are not equivalent. Unmodified, acetylated, and Mut1:

Flt1($1-3_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF

binding, whereas Mut4: Flt1($1-3_{R->N}$)-Fc is somewhat less efficient at

blocking binding

20

Figure 18. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1($1-3_{\Delta B}$)-Fc,

Mut2: Flt1($2-3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins to Matrigel®

coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these

wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the

25   Mut1: Flt1($1-3_{\Delta B}$)-Fc protein binds more weakly still, and the Mut2:

Flt1($2-3_{\Delta B}$)-Fc protein shows the best profile, binding more weakly

than any of the other mutant proteins. The Mut4: Flt1($1-3_{R->N}$)-Fc

glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

Figure 19. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc,

5   Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

10   **Figure 20.** Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-3$_{\Delta B}$)-Fc - 0.7μg/ml.

15

Figure 21A-21C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

Figure 22A-22C. Nucleotide and deduced amino acid sequence of the

20   modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Figure 23. Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the

25   ECM as compared to the Flt1(1-3)-Fc protein.

Figure 24A-24C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Figure 25A-25C. Phosphorylation assay. At a 1.5 molar excess of

5   either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient

Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor

stimulation by these three modified Flt1 receptors as compared to

control media challenge.   In contrast, transient

Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this

10   molar excess, as compared with VEGF positive control challenge.

Similar results are seen in Figure 25B, where the modified Flt

receptors are in a 3-fold molar excess to VEGF165 ligand.   In Figure

25C, where the modified Flt1 receptors are in a 6-fold molar excess to

VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown

15   to be partially blocking VEGF165-induced stimulation of cell-surface

receptors.

Figure 26A-26B. Phosphorylation assay.   Detection by Western blot

of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation

20   shows that cell-surface receptors are not phosphorylated by challenge

samples which have VEGF165 preincubated with 1 and 2 fold molar

excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either

transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or

transient VEGFR1R2-FcΔC1(a).   At all modified Flt1 receptor

25   concentrations tested there is complete binding of VEGF165 ligand

during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

5   **Figure 27.** MG/R2 Cell proliferation assay. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined

10 media was then added to all the wells at a concentration of 1.56nM. The negative control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking

15 VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

Figure 28. Biacore analysis of Binding Stoichiometry. Binding
20 stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule.

25

Figure 29 and Figure 30. Size Exclusion Chromatography

Stoichiometry. Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a

concentration of 1nM (estimated to be 1000 times higher than the KD of

the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165

5   interaction) were mixed with varied concentrations of VEGF165. After

incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in

solution were measured. The data shows that the addition of 1 nM

VEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocks

Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result

10   suggested the binding stoichiometry of one VEGF165 molecule per one

Flt1D2Flk1D3.FcΔC1(a) molecule.

Figure 31. Size Exclusion Chromatography (SEC) under native

conditions. Peak #1 represents the Flt1D2Flk1D3.FcΔC1(a)/ VEGF165

15   complex and peak #2 represents unbound VEGF165. Fractions eluted

between 1.1 and 1.2 ml were combined and guanidinium hydrochloride

(GuHCl)was added to a final concentration 4.5M to dissociate the

complex.

20   **Figure 32.** Size Exclusion Chromatography (SEC) under dissociative

conditions. To separate the components of the receptor-ligand complex

and to determine their molar ratio, 50µl of dissociated complex was

loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and

eluted. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2

25   represents VEGF165.

24

Figure 33, Figure 34 and Figure 35. Size Exclusion

Chromatography (SEC) with On-Line Light Scattering. Size exclusion

chromatography column with a MiniDawn on-line light scattering

5   detector (Wyatt Technology, Santa Barbara, California) and refractive

index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine

the molecular weight (MW) of the receptor-ligand complex. As shown in

Figure 33, the elution profile shows two peaks. Peak #1 represents the

receptor-ligand complex and peak #2 represents the unbound VEGF165.

10   MW was calculated from LS and RI signals. The same procedure was

used to determine MW of the individual components of the receptor-

ligand complex. The results of these determinations are as follows:

MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak

position is 157 300 (Figure 33), the MW of VEGF165 at the peak

15   position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113

300 (Figure 35).

Figure 36. Peptide mapping and glycosylation analysis. The disulfide

structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were

20   determined by a peptide mapping method. There are a total of ten

cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc

region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded

to Cys 182. The first two cysteines in the Fc region (Cys211 and

Cys214) form an intermolecular disulfide bond with the same two

25   cysteines in another Fc chain. However, it can not be determined

whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410.

5

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) and are found to be glycosylated to varying degrees. Complete glycosylation is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites

10   of glycosylation are highlighted by underline in the Figure.

Figure 37. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a). Balb/c mice were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO

15   transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-

20   FcΔC1(a). The Tmax for Flt1(1-3)-Fc (A40) was at 6 hrs while the Tmax for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The Cmax for Flt1(1-3)-Fc (A40) was 8μg/ml, For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the Cmax was 18μg/ml and the Cmax for the

25   stable VEGFR1R2-FcΔC1(a) was 30μg/ml.

26

Figure 38. Pharmacokinetics of Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Balb/c mice

were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO

5   transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently

expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2,

5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in

an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and

Flt1D2.VEGFR3D3.FcΔC1(a). Flt1(1-3)-Fc (A40) could no longer be

10   detected in the serum after day 5 whereas Flt1D2.Flk1D3.FcΔC1(a) and

Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more.

Figure 39. The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit HT-1080

Fibrosarcoma Tumor Growth In Vivo. Every other day or 2 times per

15   week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) at 25mg/Kg

significantly decreases the growth of subcutaneous HT-1080

fibrosarcoma tumors.

Figure 40. The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit C6 Glioma

20   Tumor Growth In Vivo. Every other day or 2 times a week treatment of

SCID mice with Flt1D2.Flk1D3.FcΔC1(a) significantly decreases the

growth of subcutaneous C6 glioma tumors at doses as low as 2.5mg/Kg.

Figure 41. VEGF-Induced Uterine Hyperpermeability. PMSG injected

25   subcutaneously (5 IU) to induce ovulation in prepubertal female rats

results in a surge of estradiol after 2 days which in turn causes an

induction of VEGF in the uterus.   This induction results in

hyperpermeability of the uterus and an increase in uterine wet.

Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a)

5   and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr after PMSG injection

results in about a 50% inhibition of the increase in uterine wet weight.

Figure 42A-42B.   Assessment of Corpus Luteum Angiogenesis Using

Progesterone as a Readout.   PMSG was injected subcutaneously (5 IU) to

10   induce ovulation in prepubertal female rats, resulting in a fully

functioning corpus luteum containing a dense network of blood vessels

that secretes progesterone into the blood stream to prepare the uterus

for implantation.   The induction of angiogenesis in the corpus luteum

requires VEGF.   Resting levels of progesterone are about 5ng/ml and can

15   be induced to 25-40ng/ml after PMSG.   Subcutaneous injection of

Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at

1hr. after PMSG injection resulted in a complete inhibition of the

progesterone induction on day 4.

20   **DETAILED DESCRIPTION OF THE INVENTION**

It has been a long standing problem in the art to produce a receptor

based VEGF antagonist that has a pharmacokinetic profile that is

appropriate for consideration of the antagonist as a therapeutic

25   candidate.   Applicants describe herein, for the first time, a chimeric

polypeptide molecule, capable of antagonizing VEGF activity, that

exhibits improved pharmacokinetic properties as compared to other

known receptor-based VEGF antagonists. The chimeric polypeptide

molecules described herein thus provide for the first time appropriate

5   molecules for use in therapies in which antagonism of VEGF is a desired

result.

The present invention provides for novel chimeric polypeptide

molecules formed by fusing a modified extracellular ligand binding

10   domain of the Flt1 receptor to the Fc region of IgG.

The extracellular ligand binding domain is defined as the portion of a

receptor that, in its native conformation in the cell membrane, is

oriented extracellularly where it can contact with its cognate ligand.

15   The extracellular ligand binding domain does not include the

hydrophobic amino acids associated with the receptor's transmembrane

domain or any amino acids associated with the receptor's intracellular

domain. Generally, the intracellular or cytoplasmic domain of a

receptor is usually composed of positively charged or polar amino acids

20   (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The

preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e.

leucine, valine, isoleucine, and phenylalanine) comprise the

transmembrane domain. The extracellular domain comprises the amino

acids that precede the hydrophobic transmembrane stretch of amino

25   acids. Usually the transmembrane domain is flanked by positively

29

charged or polar amino acids such as lysine or arginine. von Heijne has

published detailed rules that are commonly referred to by skilled

artisans when determining which amino acids of a given receptor

belong to the extracellular, transmembrane, or intracellular domains

5   (See von Heijne, 1995, BioEssays 17:25-30). Alternatively, websites on

the Internet, such as

http://ulrec3.unil.ch/software/TMPRED_form.html. have become

available to provide protein chemists with information about making

predictions about protein domains.

10

The present invention provides for the construction of nucleic acid

molecules encoding chimeric polypeptide molecules that are inserted

into a vector that is able to express the chimeric polypeptide molecules

when introduced into an appropriate host cell. Appropriate host cells

15   include, but are not limited to, bacterial cells, yeast cells, insect cells,

and mammalian cells. Any of the methods known to one skilled in the

art for the insertion of DNA fragments into a vector may be used to

construct expression vectors encoding the chimeric polypeptide

molecules under control of transcriptional/translational control

20   signals. These methods may include in vitro recombinant DNA and

synthetic techniques and in vivo recombinations (genetic

recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory

Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular

Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience,

25   NY).

Expression of nucleic acid molecules encoding the chimeric polypeptide
molecules may be regulated by a second nucleic acid sequence so that
the chimeric polypeptide molecule is expressed in a host transformed

5   with the recombinant DNA molecule. For example, expression of the
chimeric polypeptide molecules described herein may be controlled by
any promoter/enhancer element known in the art. Promoters which may
be used to control expression of the chimeric polypeptide molecules
include, but are not limited to, the long terminal repeat as described in

10   Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region
(Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter,
the M-MuLV 5' terminal repeat the promoter contained in the 3' long
terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell
22:787-797), the herpes thymidine kinase promoter (Wagner et al.,

15   1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory
sequences of the metallothionine gene (Brinster et al., 1982, Nature
296:39-42); prokaryotic expression vectors such as the β-lactamase
promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A.
75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl.

20   Acad. Sci. U.S.A. 80:21-25, see also "Useful proteins from recombinant
bacteria" in Scientific American, 1980, 242:74-94); promoter elements
from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol
dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter,
alkaline phosphatase promoter, and the following animal

25   transcriptional control regions, which exhibit tissue specificity and

31

have been utilized in transgenic animals: elastase I gene control region

which is active in pancreatic acinar cells (Swift et al., 1984, Cell

38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol.

50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene

5   control region which is active in pancreatic beta cells (Hanahan, 1985,

Nature 315:115-122), immunoglobulin gene control region which is

active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658;

Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol.

Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region

10   which is active in testicular, breast, lymphoid and mast cells (Leder et

al., 1986, Cell 45:485-495), albumin gene control region which is

active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276),

alpha-fetoprotein gene control region which is active in liver (Krumlauf

et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science

15   235:53-58); alpha 1-antitrypsin gene control region which is active in

the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin

gene control region which is active in myeloid cells (Mogram et al.,

1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin

basic protein gene control region which is active in oligodendrocyte

20   cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light

chain-2 gene control region which is active in skeletal muscle (Shani,

1985, Nature 314:283-286), and gonadotropic releasing hormone gene

control region which is active in the hypothalamus (Mason et al., 1986,

Science 234:1372-1378).

25

32

Thus, according to the invention, expression vectors capable of being

replicated in a bacterial or eukaryotic host comprising chimeric

polypeptide molecule-encoding nucleic acid as described herein, are

used to transfect the host and thereby direct expression of such nucleic

5   acids to produce the chimeric polypeptide molecules, which may then be

recovered in a biologically active form.   As used herein, a biologically

active form includes a form capable of binding to VEGF.

Expression vectors containing the chimeric nucleic acid molecules

10   described herein can be identified by three general approaches: (a)

DNA-DNA hybridization, (b) presence or absence of "marker" gene

functions, and (c) expression of inserted sequences.   In the first

approach, the presence of a foreign gene inserted in an expression

vector can be detected by DNA-DNA hybridization using probes

15   comprising sequences that are homologous to the inserted chimeric

polypeptide molecule sequences.   In the second approach, the

recombinant vector/host system can be identified and selected based

upon the presence or absence of certain "marker" gene functions (e.g.,

thymidine kinase activity, resistance to antibiotics, transformation

20   phenotype, occlusion body formation in baculovirus, etc.) caused by the

insertion of foreign genes in the vector.   For example, if the chimeric

polypeptide molecule DNA sequence is inserted within the marker gene

sequence of the vector, recombinants containing the insert can be

identified by the absence of the marker gene function.   In the third

25   approach, recombinant expression vectors can be identified by assaying

33

the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

5   Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique

10   which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for

15   example, Builder, et al., US Patent No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

20   In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further

25   embodiments of the invention may be prepared in which the order of the

34

first, second and third fusion polypeptide components are rearranged.
For example, if the nucleotide sequence encoding the first component is
designated 1, the nucleotide sequence encoding the second component is
designated 2, and the nucleotide sequence of the third component is

5   designated 3, then the order of the components in the isolated nucleic
acid of the invention as read from 5' to 3' may be any of the following
six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

The present invention also has diagnostic and therapeutic utilities.   In

10   particular embodiments of the invention, methods of detecting
aberrancies in the function or expression of the chimeric polypeptide
molecules described herein may be used in the diagnosis of disorders.
In other embodiments, manipulation of the chimeric polypeptide
molecules or agonists or antagonists which bind the chimeric

15   polypeptide molecules may be used in the treatment of diseases.   In
further embodiments, the chimeric polypeptide molecule is utilized as
an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may

20   be useful in treating clinical conditions that are characterized by
vascular permeability, edema or inflammation such as brain edema
associated with injury, stroke or tumor; edema associated with
inflammatory disorders such as psoriasis or arthritis, including
rheumatoid arthritis; asthma; generalized edema associated with burns;

25   ascites and pleural effusion associated with tumors, inflammation or

35

trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

5

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine. An IEF analysis of Flt1(1-3)-Fc showed that this protein has pI greater than 9.3, confirming the prediction that the protein is very basic. It

10 was hypothesized that the basic nature of Flt1(1-3)-Fc protein was causing it to bind to extracellular matrix components and that this interaction might be the cause of the extremely short detectable circulating serum half-life exhibited by Flt1(1-3)-Fc when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was

15 acetylated at the lysine residues to reduce the basic charge. Acetylated Flt1(1-3)-Fc was then tested in the assays described *infra*.

The following examples are offered by way of illustration and not by way of limitation.

20   **EXAMPLES**

Example 1: Expression of Flt1(1-3)-Fc protein in CHO K1 cells.

Using standard molecular biology techniques (see e.g., Molecular
25 Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor

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Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al.,

Greene Publ. Assoc., Wiley-Interscience, NY), the gene encoding Flt1(1-

3)-Fc was inserted into the expression vector pEE14.1 (Lonza Biologics,

plc) at a multiple cloning site downstream of the CMV promoter. CHO

5   K1 cells were transfected with the pEE14.1/Flt1(1-3)-Fc DNA

construct using lipofectamine (Gaithersburg, MD). The transfected CHO

K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, MO)

containing 25µM methionine sulfoximine (MSX) from Sigma Inc., St.

Louis, MO, and high recombinant protein expressors were obtained by

10   screening the CHO K1 cell supernatants from over 100 hand-picked

colony isolates using a standard immunoassay which captures and

detects human Fc. The selected hand-picked clone was amplified in the

presence of 100 µM MSX followed by a second round of screening of the

amplified clones. The highest producing clone had a specific

15   productivity of recombinant Flt1(1-3)-Fc protein of 55 pg/cell/day.

The selected clone was expanded in 225cm^2 T-flasks (Corning, Acton,

MA) and then into 8.5L roller bottles (Corning, Acton, MA) using the cell

culture media described *supra*. Cells were removed from the roller

20   bottles by standard trypsinization and put into 3.5L of suspension

medium. The suspension medium is comprised of glutamine-free ISCHO

medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal bovine

serum (FBS from Hyclone Labs, Logan, UT), 100µM MSX and GS

supplement (JRH Scientific, Kansas City, MO) in a 5L Celligen

25   bioreactor (New Brunswick Scientific, New Brunswick, NJ) at a density

37

of 0.3 x 10^6 cells/mL. After the cells reached a density of 3.6 x

10^6/mL and were adapted to suspension they were transferred to a 60L

bioreactor (ABEC, Allentown, PA) at a density of 0.5 x 10^6 cells/mL in

20L of ISCHO medium with 5% fetal bovine serum. After two days an

5   additional 20L of ISCHO + 5% fetal bovine serum was added to the

bioreactor. The cells were allowed to grow for an additional two days

reaching a final density of 3.1 x 10^6 cells/mL, and a final Flt1(1-3)-Fc

concentration at harvest was 95 mg/L. At harvest the cells were

removed by tangential flow filtration using 0.45μm Prostak Filters

10   (Millipore, Inc., Bedford, MA).

Example 2: Purification of Flt1(1-3)-Fc protein obtained from CHO K1 cells

15   Flt1(1-3)-Fc protein was initially purified by affinity chromatography.

A Protein A column was used to bind, with high specificity, the Fc

portion of the molecule. This affinity-purified protein was then

concentrated and passed over a SEC column. The protein was then

eluted into the formulation buffer. The following describes these

20   procedures in detail.

Materials and Methods

All chemicals were obtained from J.T. Baker, Phillipsburg, NJ with the

25   exception of PBS; which was obtained as a 10X concentrate from Life

Technologies, Gaithersburg, MD. Protein A Fast Flow and Superdex 200

preparation grade resins were obtained from Pharmacia, Piscataway,

NJ. Equipment and membranes for protein concentration were obtained

from Millipore, Bedford, MA.

5

Approximately 40L of 0.45μm-filtered CHO conditioned media

containing Flt1(1-3)-Fc protein was applied to a 290mL Protein A Fast

Flow column (10cm diameter) that had been equilibrated with PBS. The

column was washed with PBS containing 350mM NaCl and 0.02% CHAPS

10   and the bound protein was eluted with 20mM Citric Acid containing

10mM Na_2HPO_4. The single peak in the elution was collected and its pH

was raised to neutrality with 1M NaOH. The eluate fractions was

concentrated to approximately 9 mg/mL using 10K regenerated

cellulose membranes by both tangential flow filtration and by stirred

15   cell concentration. To remove aggregates and other contaminants, the

concentrated protein was applied to a column packed with Superdex 200

preparation grade resin (10cm x 55cm) and run in PBS containing 5 %

glycerol. The main peak fractions were pooled, sterile filtered,

aliquoted and stored at -80°C.

20

Example 3: Acetylation of Flt1(1-3)-Fc protein.

Two milligrams of Flt1(1-3)-Fc protein were acetylated as described

in the instruction manual provided with the sulfo-NHS-acetate

25   modification kit (Pierce Chemical Co., Rockford, IL, Cat.#26777).

Example 4: Characterization of acetylated Flt1(1-3)-Fc protein.

5   (a.) IEF analysis: Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by standard IEF analysis. As shown in Figure 1, Flt1(1-3)-Fc protein is not able to migrate into the gel and therefore must have a pI greater than 9.3, the highest pI in the standard. However, acetylated Flt1(1-3)-Fc is able to migrate into the gel and equilibrate at a pI of

10 approximately 5.2. This result demonstrates that acetylation reduces the net positive charge of the protein and therefore its pI considerably.

(b.) Binding to extracellular matrix components

15 To test for binding to extracellular matrix components, Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc where tested in an assay designed to mimic the interaction with extracellular matrix components. In this assay, 96-well tissue culture plates are coated with Matrigel (Biocoat MATRIGEL® matrix thin layer 96 well plate, Catalog #40607, Becton

20 Dickinson Labware, Bedford, MA). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. The plates are incubated for 1-2 hours at either room temperature or 37°C degrees and then detection of bound proteins is accomplished by adding

25 a secondary alkaline phosphatase-conjugated anti-human Fc antibody to

the wells. Finally, alkaline phosphatase substrate is added to the wells

and optical density is measured. Figure 2 shows the results of this

assay. Like the irrelevant control protein rTie2-Fc, acetylated Flt1(1-

3)-Fc does not exhibit any binding to the Matrigel coated plate, whereas

5   the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding.

This result indicates that acetylation of basic amino acid residues is

an effective way to interfere with the charge interactions that exist

between positively charged proteins and the negatively charged

extracellular matrix components they are exposed to *in vivo*.

10

Example 5: Pegylation of Flt1(1-3)-Fc protein.

Although pegylation (polyethylene glycol - PEG) of proteins has been

shown to increase their *in vivo* potency by enhancing stability and

15   bioavailability while minimizing immunogenicity (see references cited

supra), it is counter-intuitive that pegylating molecules that are too

large to be filtered by the kidney glomeruli would improve their

pharmacokinetic properties. Without being bound by theory, Applicants

postulated that pegylation of the Flt1(1-3)-Fc molecules could improve

20   the pharmacokinetic properties, possibly not by altering the positive

charge or by decreasing the pI of Flt1(1-3)-Fc, but rather by physically

shielding the positive charges from interacting with the extracellular

matrix. Applicants decided to attempt to improve the pharmacokinetic

properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs

25   as described *infra*.

41

Materials and Methods

Purified Flt1(1-3)-Fc derived from CHO cells (see *supra*) was used in

5   the following pegylation experiments.   Functionalized PEGs were

obtained from Shearwater Polymers, Huntsville, AL; Bicine from Sigma,

St Louis, MO; Superose 6 column from Pharmacia, Piscataway, NJ; PBS

as a 10X concentrate from Life Technologies, Gaithersburg, MD; Glycerol

from J.T. Baker, Phillipsburg, NJ; and Bis-Tris precast gels from Novex,

10 CA.

20K PEG strands functionalized with amine-specific terminal moieties

were used in small-scale reaction studies that were set-up to evaluate

different reaction conditions in which the PEG:protein stoichiometry

15 was varied.   Based on these reactions and the analyses of samples on

standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was

reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate)

molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The

reaction was allowed to proceed at 8°C overnight.   For initial

20 purification, the reaction products were applied to a 10mm x 30cm

Superose 6 column equilibrated with PBS containing 5% Glycerol.   The

column appeared to separate pegylated Flt1(1-3)-Fc molecules based on

the extent of pegylation.   Fractions corresponding to what appeared to

be primarily mono-pegylated and di-pegylated dimeric Flt1(1-3)-Fc, as

25 judged by banding patterns on reducing and non-reducing SDS-PAGE gels

were pooled.   The protein concentration was determined by measuring absorbance at 280 nm.   The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at -40°C.

5   **Example 6: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in a Biacore-based assay.**

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a Biacore-based assay to evaluate their ability to bind to the

10   Flt1 ligand, VEGF.   In this assay, unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a sample containing 0.2 µg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25 µg/ml)

15   was passed over the Flt1(1-3)-Fc-coated chip.   To minimize the effects of non-specific binding, the bound samples were washed with a 0.5M NaCl wash.   In one sample, unmodified Flt1(1-3)-Fc was mixed with heparin.   Heparin is a negatively charged molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules

20   are mixed together, they should interact through their respective charges.   This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions.   As shown in Figure 3, acetylated (columns 13-

25   16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc

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(columns 21-24) are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with

5   Biacore chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M NaCl (columns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

10

Example 7: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in an ELISA-based assay.

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were

15   tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. As shown in Figure 4, both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that modifying the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

20

Example 8: Pharmacokinetic analysis of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc.

In vivo experiments were designed to assess the pharmacokinetic

25   profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and

pegylated Flt1(1-3)-Fc protein.   Balb/c mice (23-28g; 3 mice/group) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc.   The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein.   The sera were

5   assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein.   Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc antibody linked to alkaline phosphatase.   As shown in Figure 5, the Tmax for all of the

10   Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The Cmax for the different proteins was as follows: Unmodified: 0.06 μ/ml - 0.15 μg/ml; acetylated: 1.5 μg/ml - 4.0 μg/ml; and pegylated: approximately 5 μg/ml.

15   **Example 9: Step-acetylation of Flt1(1-3)-Fc**

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the Flt1(1-3)-Fc protein in a step-wise

20   fashion by using increasing amounts of molar excess of acetylation reagent in the acetylation reaction mixture.   The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80 , 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer.   The reactions were performed as detailed in the instruction manual provided with the

sulfo-NHS-Acetate modification kit (Pierce Chemical Co., Rockford, IL, Cat.# 26777).

Example 10: Characterization of step-acetylated Flt1(1-3)-Fc.

(a.) IEF analysis   Unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins were analyzed by standard IEF analysis. As shown in Figure 6A-6B, unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely high pI (greater than 9.3). However, most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation of the protein. This result demonstrates that acetylation can change the positive charge of the protein in a dose-dependent manner and that reduction of the pI can be controlled by controlling the degree of acetylation.

(b.) Binding of step-acetylated Flt1(1-3)-Fc to extracellular matrix components

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc where tested in the above-described assay designed to mimic the interaction with extracellular matrix components. Varying concentrations of either unmodified Flt1(1-3)-Fc,

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step-acetylated Flt1(1-3)-Fc (10, 20, and 30 fold molar excess

samples), or rTie2-Fc (an irrelevant control) protein were added to the

wells. The plates were incubated for 1-2 hours at room temperature or

37°C and then detection of bound proteins was accomplished by adding a

5   secondary alkaline phosphatase-conjugated anti-human Fc antibody to

the wells. Alkaline phosphatase substrate was subsequently added to

the wells and optical density measured. Figure 7 shows the results of

this assay. Like the irrelevant control protein rTie2-Fc, step-

acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not

10   exhibit any significant binding to the Matrigel coated plate, whereas

the non-acetylated Flt1(1-3)-Fc protein exhibited significant binding.

The binding is saturable, indicating that the Flt1(1-3)-Fc protein may

be binding to specific sites, rather than a more general charge-

mediated interaction that might not be saturable. The 10 fold molar

15   excess sample showed reduced binding, but the degree of acetylation

was not enough to completely block binding to extracellular matrix

components. The 20 fold molar excess and higher samples displayed no

detectable binding, despite the fact that by IEF analysis (Figure 6A and

6B) the lower molar excess samples still had a large net positive

20   charge. This result demonstrates that it is not necessary to completely

acetylate all available basic amino acids in order to eliminate binding

to extracellular matrix components.

(c.) Binding of step-acetylated Flt1(1-3)-Fc in a Biacore-based assay.

Unmodified and step-acetylated Flt1(1-3)-Fc proteins where tested in

5   a Biacore-based assay to evaluate their ability to bind to the Flt1

ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0,

or 5.0 µg/ml) was immobilized on the surface of a Biacore chip (see

Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for

standard procedures) and a solution containing 0.2 µg/ml VEGF and

10   either unmodified Flt1(1-3)-Fc (at either 0.5, 1.0, or 5.0 µg/ml) or 10

different step-acetylated Flt1(1-3)-Fc samples (at 0.5, 1.0, or 5.0

µg/ml each) were passed over the Flt1(1-3)-Fc-coated chip. As shown

in Figure 8, at a sub-stoichiometric ratio (0.5 µg/ml of either

unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml

15   VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-

acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml,

which approximates a 1:1 stoichiometric ratio, both unmodified and

step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF

binding, but there is still insufficient Flt1(1-3)-Fc protein (either

20   unmodified or step-acetylated) to completely bind the available VEGF.

However, at 5.0 µg/ml, which is several times greater than a 1:1

stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated

Flt1(1-3)-Fc proteins are able to bind the VEGF, regardless of the

degree of acetylation. This clearly demonstrates that acetylation does

25   not alter Flt1(1-3)-Fc's ability to bind VEGF.

(d.) Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic

5   profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc

protein.   Balb/c mice (23-28g) were injected subcutaneously with

4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess

samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20

and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar

10   excess samples).   The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days

and 3 days after injection.   The sera were assayed in an ELISA-based

assay designed to detect Flt1(1-3)-Fc (described *supra*).   Figure 9

details the results of this study.   The Tmax for all of the Flt1(1-3)-Fc

proteins tested was at the 6 hour time point but the Cmax was as

15   follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold molar excess

sample: - 0.7μg/ml, 20 fold molar excess sample - 2μg/ml, 40 fold

molar excess sample - 4μg/ml, 60 fold molar excess sample - 2μg/ml,

100 fold molar excess sample - 1μg/ml.   This results demonstrates

that acetylation or pegylation of Flt1(1-3)-Fc significantly improves

20   its pharmacokinetic profile.

Example 11: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

25   Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI

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below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix

5   components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components.   For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues.   Sometimes these residues are found in a cluster on the

10   primary sequence of the protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example Hileman, et al., 1998, Bioessays 20(2):156-67).   In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come

15   from different regions of the primary sequence and are only brought together when the protein folds into its tertiary structure.   Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein.   However, if there is a cluster of positively charged amino acid residues in the

20   primary sequence, it is not unreasonable to surmise that the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site.   Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876).   Referring

25   to the nucleic acid and amino acid sequence set forth in Figure 10A-10D

of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78.   The mature protein begins with Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid

5   sequence.   Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr.   Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala).   There is a bridging amino acid sequence, Gly-Pro-Gly,

10   encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that

15   there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of Figure 10A-10D, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand.   An alignment of the

20   sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic

25   region in the protein (Figure 12A-12B).   These observations raised the

possibility that the actual three dimensional conformation of Flt1 Ig

domain 3 allowed for some type of protrusion that is not in Flt1 Ig

domain 2. To test this hypothesis, the 10 additional amino acids were

deleted and the resulting protein was tested to see whether the

5   deletion would affect the pharmacokinetics favorably without seriously

compromising the affinity of the receptor for VEGF. This DNA

construct, which was constructed using standard molecular biology

techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook,

et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular

10   Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience,

NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc.,

Cambridge, MA), is referred to as Mut1: Flt1(1-3$_{\Delta B}$)-Fc. The Mut1:

Flt1(1-3$_{\Delta B}$)-Fc construct was derived from Flt1(1-3)-Fc by deletion of

nucleotides 814-843 (set forth in Figure 10A-10D), which deletes the

15   highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-

Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA

sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied

20   Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3$_{\Delta B}$)-

Fc is set forth in Figure 13A-13D.

Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3 $_{\Delta B}$)-Fc.

A second deletion mutant construct, designated Mut2: Flt1(2-3$_{\Delta B}$)-Fc,

5   was derived from the Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct by deletion of Flt1

Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D); for

convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA.

This introduced a restriction site (BspE1) without altering the

associated amino acid sequence, Ser-Gly. This DNA construct, which

10 was constructed using standard molecular biology techniques (see e.g.,

Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring

Harbor Laboratory), Current Protocols in Molecular Biology (Eds.

Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the

mammalian expression vector pMT21 (Genetics Institute, Inc.,

15 Cambridge, MA), was also sequence-verified using an ABI 373A DNA

sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied

Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-3$_{\Delta B}$)-

Fc is set forth in Figure 14A-14C.

20 ## Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was

constructed the same way as the Mut2: Flt1(2-3$_{\Delta B}$)-Fc construct,

25 except that Flt1 Ig domain 3 was left intact (the basic region amino

53

acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C.

5

Example 14: Construction of Flt(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3 $_{R->N}$)-Fc.

A final construct was made in which a N-glycosylation site was

10 introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). The resulting amino acid sequence is therefore changed from

15 Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D.

20 **Example 15: Characterization of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and Mut4: Flt1(1-3$_{R->N}$)-Fc mutants.**

(a.) Binding to extracellular matrix components

25 To determine whether the three modified proteins were more or less

likely to have improved pharmacokinetic properties, Matrigel coated 96-well dishes (as described *supra*) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkaline-phosphatase conjugated antibodies. As shown in Figure 18,

5 this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-Fc protein bound somewhat more weakly, the Mut1: Flt1(1-3$_{\Delta B}$)-Fc protein bound more weakly still, and the Mut2: Flt1(2-3$_{\Delta B}$)-Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The

10 Mut4: Flt1(1-3$_{R->N}$)-Fc glycosylation mutant protein showed only marginal benefit on the Matrigel assay. These results confirm the hypothesis that a linear sequence of positive amino acids can be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

15

(b.) Binding of Mut1: Flt1(1-3$_{\Delta B}$)-Fc and Mut4: Flt1(1-3$_{R->N}$)- Fc in a Biacore-based assay.

Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1:

20 Flt1(1-3$_{\Delta B}$)-Fc and Mut4: Flt1(1-3$_{R->N}$)-Fc proteins where tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.25, 0.5, or 1.0 μg/ml) was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard

25 procedures) and a solution containing 0.1 μg/ml VEGF and either

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purified or COS cell supernatant containing unmodified Flt1(1-3)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0 μg/ml), COS cell supernatant containing Mut1: Flt1(1-3$_{\Delta B}$)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml), or COS cell

5   supernatant containing Mut4: Flt1(1-3$_{R->N}$)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in Figure 17, at the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. μg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block

10   binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc

15   proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3$_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3$_{R->N}$)-Fc is somewhat less efficient at

20   blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

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(c.) <u>Binding of Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc,</u>
<u>Mut3: Flt1(2-3)-Fc, and in an ELISA-based assay.</u>

To determine whether the three mutant proteins could bind the Flt1

5   ligand VEGF, binding experiments were done in which 96-well plates

coated with VEGF were incubated with varying concentrations of the

respective mutant protein, and after washing, the amount bound was

detected by incubating with an alkaline phosphatase conjugated anti-

human Fc antibody and quantitated colorimetrically by the addition of

10   an appropriate alkaline phosphatase substrate.   As shown in Figure 19,

this experiment showed that all the mutant proteins could bind VEGF

similarly, at the concentrations tested.

Example 16: Pharmacokinetic analysis of acetylated Flt1(1-

15   **3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and unmodified Flt1(1-3)-Fc.**

In vivo experiments were designed to assess the pharmacokinetic

profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and 40 fold

molar excess acetylated Flt1(1-3)-Fc protein.   Balb/c mice (25-30g)

20   were injected subcutaneously with 4mg/kg of unmodified Flt1(1-3)-Fc,

40 fold molar excess acetylated Flt1(1-3)-Fc, and Mut1: Flt1(1-3$_{\Delta B}$)-Fc

proteins (4 mice each).   These mice were tail bled at 1, 2, 4, 6, 24

hours, 2 days, 3 days, and 5 days after injection.   The sera were

assayed in an ELISA designed to detect Flt1(1-3)-Fc protein which

25   involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and

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reporting with an anti-Fc antibody linked to alkaline phosphatase. As

shown in Figure 20, the Cmax for these reagents was as follows:

Unmodified Flt1(1-3)-Fc - 0.15µg/ml; 40 fold molar excess acetylated

Flt1(1-3)-Fc - 1.5µg/ml; and Mut1: Flt1(1-3$_{\Delta B}$)-Fc - 0.7µg/ml.

5

Example 17: Modified Flt1 receptor vector construction

The rationale for constructing modified versions of the Flt1 receptor

(also known as VEGFR1) was based on the observation that the protein

10   sequence of Flt1 was highly basic, and was therefore likely to stick to

extracellular matrix (ECM).   The highly basic nature of Flt1 probably

explains why unmodified Flt1(1-3)-Fc (described *supra*) has poor

pharmacokinetics that make it difficult to use as a therapeutic agent.

As described *supra*, the chemically modified form of 40 fold molar

15   excess acetylated Flt1(1-3)-Fc, hereinafter termed A40, exhibited a

greatly improved pharmacokinetic (PK) profile over the non-acetylated

Flt1(1-3)-Fc.   Therefore, attempts were made to engineer DNA

molecules that could be used to   recombinantly express modified forms

of a Flt1 receptor molecule that would possess the improved PK profile

20   exhibited by A40 and still maintain the ability to bind tightly to VEGF.

It is known in the literature that the first Ig domain of Flt1 (which has

a net charge of +5 at neutral pH) is not essential for tight binding to

VEGF, so this domain was deleted.   The third Ig domain (having a net

25   charge of +11) is not essential for binding, but confers higher affinity

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for VEGF than the second Ig domain, so instead of deleting it entirely, it

was replaced with the equivalent domains of the Flt1 receptor

relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3).

These chimeric molecules (denoted R1R2 (Flt1.D2.Flk1D3.FcΔC1(a) and

5   VEGFR1R2-FcΔC1(a) and R1R3 (Flt1D2.VEGFR3D3-FcΔC1(a) and

VEGFR1R3-FcΔC1(a) respectively, wherein R1 and Flt1D2 = Ig domain 2

of Flt1 (VEGFR1); R2 and Flk1D3 = Ig domain 3 of Flk1 (VEGFR2); and R3

and VEGFR3D3 = Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to

ECM, as judged by an *in vitro* ECM binding assay as described *infra* , had

10   greatly improved PK as described *infra*.   In addition, these molecules

were able to bind VEGF tightly as described *infra* and block

phosphorylation of the native Flk1 receptor expressed in endothelial

cells as described *infra.*

15   **(a)   Construction of the expression plasmid**

pFlt1D2.Flk1D3.FcΔC1(a)

Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flk-1(1-

3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-

20   tagged versions of Ig domains 1-3 of human Flt1 and human Flk1,

respectively. These plasmids were used to construct a DNA fragment

consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1,

using PCR amplification of the respective Ig domains followed by

further rounds of PCR to achieve fusion of the two domains into a

single fragment.   For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3')

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C).   The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of Figure 21A-21C) and continuing into VVLS (corresponding to amino acids 127-130 of Figure 21A-21C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3')

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC AAATG-3')

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (corresponding to amino acids 223-228 of

5   Figure 21A-21C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C.

10   After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and

15   the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI

20   and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C.

25

(b)   Construction of the expression plasmid

pFlt1D2VEGFR3D3FcΔC1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin

5   resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1

receptor.   This plasmid was used to produce a DNA fragment containing

Ig domain 2 of Flt1 by PCR.   RNA from the cell line HEL921.7 was used

to produce Ig domain 3 of Flk1, using standard RT-PCR methodology.   A

further round of PCR amplification was used to achieve fusion of the

10   two Ig domains into a single fused fragment.   For Ig domain 2 of Flt1,

the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

15   3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA

TTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream

of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM

20   (corresponding to amino acids 27-33 of Figure 22A-22C).   The 3'

amplification primer encodes the reverse complement of the end of Flt1

Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with

the fusion point defined as TIID of Flt1 (corresponding to amino acids

123-126 of Figure 22A-22C) and continuing into IQLL of VEGFR3

25   (corresponding to amino acids 127-130 of Figure 22A-22C).

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For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

5   5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3.
10   The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:
15

5':Flt1D2.VEGFR3D3.s

(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as
20   (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of
25   VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding

to amino acids 221-226 of Figure 22A-22C), followed by a bridging

sequence that includes a recognition sequence for Srf1, and encodes the

amino acids GPG. The bridging sequence corresponds to amino acids

227-229 of Figure 22A-22C.

5

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain

3) of PCR to produce the individual Ig domains, the PCR products were

combined in a tube and subjected to a further round of PCR

amplification with the amplification primers bsp/flt1D2 and

10   VEGFR3D3/srf.as described *supra*, to produce the fusion product. This

PCR product was subsequently digested with the restriction enzymes

BspEI and SmaI and the resulting 625bp fragment was subcloned into

the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc

(described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc.

15   The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified

by standard sequence analysis. This plasmid was then digested with

the restriction enzymes EcoRI and SrfI and the resulting 693bp

fragment was subcloned into the EcoRI to SrfI restriction sites of the

plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated

20   pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid

sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set

forth in Figure 22A-22C.

Example 18:   Extracellular Matrix Binding (ECM) Binding Assay

ECM-coated plates (Becton Dickinson catalog # 35-4607) were

rehydrated with warm DME supplemented with glutamine (2mM), 100U

5   penicillin, 100U streptomycin, and 10% BCS for at least 1 hr. before

adding samples. The plates were then incubated for 1 hr. at room

temperature with varying concentrations of Flt1D2.Flk1D3.FcΔC1(a) and

Flt1D2.VEGFR3D3.FcΔC1(a) starting at 10nM with subsequent 2-fold

dilutions in PBS plus 10% BCS. The plates were then washed 3 times

10   with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-

conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10%

BCS) for 1 hr. at room temperature. The plates were then washed 4

times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP

solution (Sigma) was added for color development. Plates were read at

15   I = 405-570nm. The results of this experiment are shown in Figure 23

and demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and

Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the

ECM as compared to the Flt1(1-3)-Fc protein.

20   ## Example 19:   Transient expression of pFlt1D2.Flk1D3.FcΔC1(a)
in CHO-K1 (E1A) cells

A large scale (2L) culture of E. coli DH10B cells carrying the

pFlt1D2.Flk1D3.FcΔC1(a) plasmid described *supra* in Example 17(a) was

25   grown overnight in Terrific Broth (TB) plus 100μg/ml ampicillin. The

65

next day, the plasmid DNA was extracted using a QIAgen Endofree

Megaprep kit following the manufacturer's protocol.   The concentration

of the purified plasmid DNA was determined by standard techniques

using a UV spectrophotometer and fluorometer.   The plasmid DNA was

5   verified by standard restriction enzyme digestion of aliquots using the

restriction enzymes EcoRI plus NotI and AseI.   All restriction enzyme

digest fragments corresponded to the predicted sizes when analyzed on

a 1% agarose gel.

10   Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a

density of 4 x 106 cells/plate.   Plating media was Gibco Ham's F-12

supplemented with 10% Hyclone Fetal Bovine Serum (FBS), 100U

penicillin/100U streptomycin and glutamine (2mM).   The following day

each plate of cells was transfected with 6 μg of the

15   pFlt1D2.Flk1D3.FcΔC1(a) plasmid DNA using Gibco Optimem and Gibco

Lipofectamine in 12 ml volume, following the manufacturer's protocol.

Four hours after adding the transfection mix to the cells, 12 ml/plate

of Optimem supplemented with 10% FBS was added.   Plates were

incubated at 37°C in a 5% CO_2 incubator overnight.   The following day

20   the media was removed from each plate and 25 ml expression media

(Gibco CHO-S-SFM II supplemented with glutamine (2mM) and 1mM

sodium butyrate) was added.   The plates were incubated at 37°C for 3

days.   After 3 days of incubation, the media was aspirated from each

plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet

cells.   The supernatant was decanted into sterile 1L bottles and purification of the expressed protein was performed as described *infra*.

Example 20:   Construction pVEGFR1R2-FcΔC1(a) expression

5   **vector**

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C) between Flt1d2-Flk1d3-FcΔC1(a)

10   amino acids 26 and 27 of Figure 21A-21C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure.   The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing.   The GPG (bridging sequence) was removed so that

15   the Flt1 and Flk1 Ig domains were fused directly to one another.   The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C.

Example 21:   Cell Culture Process Used to Produce Modified

20   **Flt1   Receptors**

(a) Cell Culture Process Used to Produce

Flt1D2.Flk1D3.FcΔC1(a)

25   The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the

expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described *supra* in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product.

The cells are grown in bioreactors and the protein product is isolated

5   and purified by affinity and size exclusion chromatography. The process is provided in greater detail below.

Cell Expansion

10   Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.FcΔC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and 5% CO_2. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added

15   to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37°C and 5% CO_2 until confluent.

20 · **Suspension Culture in Bioreactors**

Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5L bioreactor   (New Brunswick Celligen

25   Plus) where the cells are grown in 3.5L of suspension culture. The

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suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 µM methionine sulfoximine (Sigma) was added. The pH was controlled at

5   7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37°C. When a density of 4 x10^6 cells/mL was reached the cells were transferred to a

10   40L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34°C to slow cell growth and increase the relative rate of protein expression.

15   **(b) Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.FcΔC1(a)**

The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a) were used to produce Flt1D2.VEGFR3D3.FcΔC1(a).

20

Example 22:   Harvest and Purification of Modified Flt1 Receptors

(a)   Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a)

The product protein was aseptically harvested from the bioreactor
while retaining cells using Millipore Prostak tangential-flow filtration

5   modules and a low-shear mechanical pump (Fristam). Fresh medium
was added to the bioreactor to replace that removed during the harvest
filtration.   Approximately 40L of harvest filtrate was then loaded onto
a 400 mL column containing Protein A Sepharose resin (Amersham
Pharmacia).   After loading the resin was washed with buffer containing

10   10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any
unbound contaminating proteins.   Flt1D2.Flk1D3.FcΔC1(a) protein was
eluted with a pH 3.0 citrate buffer.   The eluted protein was neutralized
by addition of Tris base and frozen at -20°C.

15   Several frozen lots of Flt1D2.Flk1D3.FcΔC1(a) protein from the Protein
A step above were thawed, pooled and concentrated using a Millipore
30kD nominal molecular weight cutoff (NMWCO) tangential flow
filtration membrane.   The protein was transferred to a stirred cell
concentrator (Millipore) and further concentrated to 30 mg/mL using a

20   30kD NMWCO membrane. The concentrated protein was loaded onto a
size exclusion column packed with Superdex 200 resin (Amersham
Pharmacia) that was equilibrated with phosphate buffered saline plus
5% glycerol. The same buffer was used to run the column. The
fractions corresponding to Flt1D2.Flk1D3.FcΔC1(a) dimer were pooled,

25   sterile filtered through a 0.22 micron filter, aliquoted and frozen.

(b) Harvest and Purification of Flt1D2.VEGFR3D3.FcΔC1(a)

The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a)

5   were used to harvest and purify Flt1D2.VEGFR3D3.FcΔC1(a).

Example 23:   Phosphorylation Assay for Transiently Expressed VEGFR2

10   Primary human umbilical vein endothelial cells (HUVECs), passage 4-6,

were starved for 2 hrs in serum-free DME high glucose media. Samples

containing 40 ng/ml (1nM) human VEGF165, which is a ligand for the

VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were

preincubated for 1 hr. at room temperature with varying amounts of the

15   modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40),

Flt1D2Flk1D3.FcΔC1(a) and Flt1D2VEGFR3D3.FcΔC1(a) in serum-free

DME-high glucose media containing 0.1% BSA. Cells were challenged for

5 minutes with the samples prepared above +/- VEGF165, followed by

whole cell lysis using complete lysis buffer. Cell lysates were

20   immunoprecipitated with an antibody directed against the C-terminus

of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto

4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane

using standard transfer methodologies. Detection of phosphorylated

VEGFR2 was done by immunoblotting with the anti-phospho Tyrosine

25   mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham).

Figures 25A-25C and 26A-26B show the results of this experiment.

Figure 25A-25C reveals that detection by Western blot of tyrosine

phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that

cell-surface receptors are phosphorylated to varying levels depending

5   on which modified Flt1 receptor is used during the preincubations with

VEGF.   As is seen in Figure 25A, at a 1.5 molar excess of either Flt1(1-

3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is

complete blockage of receptor stimulation by these three modified

Flt1 receptors as compared to control media challenge.   In contrast,

10   transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage

at this molar excess, as compared with VEGF positive control challenge.

Similar results are seen in Figure 25B, where the modified Flt

receptors are in a 3-fold molar excess to VEGF165 ligand.   In Figure

25C, where the modified Flt1 receptors are in a 6-fold molar excess to

15   VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown

to be partially blocking VEGF165-induced stimulation of cell-surface

receptors.

In Figure 26A-26B, detection by Western blot of tyrosine

20   phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that

cell-surface receptors are not phosphorylated by challenge samples

which have VEGF165 preincubated with 1 and 2 fold molar excess

(Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either

transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or

25   transient VEGFR1R2-FcΔC1(a).   At all modified Flt1 receptor

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concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

5

Example 24:   Cell Proliferation Bioassay

The test cell population is MG87 cells that have been stably transfected with a expression plasmid that contains a DNA insert encoding the

10 VEGFR2(Flk1) extracellular domain fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative

15 response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2(Flk1) to take advantage of this proliferative

20 response capability.

5×10^3 cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37°C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an

25 irrelevant receptor termed Tie2-Fc as a negative control, were titrated

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from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56nM. The plates were incubated for 72 hrs at 37°C and then MTS (Owen's reagent, Promega) added and the plates were

5   incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570nm. The results of this experiment are shown in Figure 27. The control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal

10   dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

15   **Example 25:   Binding Stoichiometry of Modified Flt Receptors to VEGF165**

(a) BIAcore Analysis

20   The stoichiometry of Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.FcΔC1(a) or

25   VEGFR1R2-FcΔC1(a) to VEGF BIAcore chip surface.

Modified Flt receptors Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a),

were captured with an anti-Fc specific antibody that was first

immobilized on a Biacore chip (BIACORE) using amine-coupling

5   chemistry. A blank antibody surface was used as a negative control.

VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over

the Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10

μl/min for one hour. A real-time binding signal was recorded and

saturation binding was achieved at the end of each injection. Binding

10   stoichiometry was calculated as a molar ratio of bound VEGF165 to the

immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the

conversion factor of 1000 RU equivalent to 1 ng/ml. The results

indicated binding stoichiometry of one VEGF165 dimeric molecule per

one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule (Figure

15   28).

In solution, Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a

concentration of 1nM (estimated to be 1000 times higher than the KD of

the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165

20   interaction) were mixed with varied concentrations of VEGF165. After

one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a)

in solution were measured as a binding signal to an amine-coupled

VEGF165 surface. A calibration curve was used to convert the

Flt1D2Flk1D3.FcΔC1(a) BIAcore binding signal to its molar

25   concentration. The data showed that the addition of 1 nM VEGF165 into

the Flt1D2Flk1D3.FcΔC1(a) solution completely blocked

Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface.   This result

suggested the binding stoichiometry of one VEGF165 molecule per one

Flt1D2Flk1D3.FcΔC1(a) molecule (Figure 29 and Figure 30).   When the

5   concentration of Flt1D2Flk1D3.FcΔC1(a) was plotted as a function of

added concentration of VEGF165, the slope of the linear portion was -

1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1,07 for VEGFR1R2-FcΔC1(a).   The

magnitude of the slope, very close to negative one, was indicative that

one molecule of VEGF165 bound to one molecule of either

10   Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a).

(b) Size Exclusion Chromatography

Flt1D2Flk1D3.FcΔC1(a) was mixed with a 3-fold excess of VEGF165 and

15   the receptor-ligand complex was purified using a Pharmacia Superose 6

size exclusion chromatography column.   The receptor-ligand complex

was then incubated in a buffer containing 6M guanidine hydrochloride in

order to dissociate it into its component proteins.

Flt1D2Flk1D3.FcΔC1(a) was separated from VEGF165 using Superose 6

20   size exclusion chromatography column run in 6M guanidium chloride.   In

order to determine complex stoichiometry, several injections of

Flt1D2Flk1D3.FcΔC1(a) and VEGF165 were made and peak height or peak

integrated intensity was plotted as a function of the concentration of

injected protein.   The calibration was done under condition identical to

25   one used in separating components of Flt1D2Flk1D3.FcΔC1(a)/VEGF

complex. Quantification of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in Figure 28, which shows the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

5

Example 26: Determination of the Binding Stoichiometry of Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography

10 ## Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex Preparation

VEGF165 (concentration = 3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2.Flk1D3.FcΔC1(a) (concentration = 0.9 mg/ml) in molar ratio of 3:1 (VEGF165:Flt1D2.Flk1D3.FcΔC1(a)) and

15 incubated overnight at 4°C.

(a) Size Exclusion Chromatography (SEC) under native conditions

20 To separate the complex from excess of unbound VEGF165, 50 μl of the complex was loaded on a Pharmacia Superose 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40μl/min. at room temperature. The results of this SEC are shown in Figure 31. Peak #1 represents the complex and peak

25 #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2

ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex.

(b) Size Exclusion Chromatography (SEC) under dissociative
5 ## conditions

To separate the components of the receptor-ligand complex and to determine their molar ratio, 50μl of dissociated complex as described *supra* was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M
10 GuHCl and eluted with the same solution at a flow rate 40μl/min. at room temperature. The results of this SEC are shown in Figure 32. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

15 ## (c) Calculation of Flt1D2Flk1D3.FcΔC1(a):VEGF165 Complex
Stoichiometry

The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of
20 VEGF165 and Flt1D2Flk1D3.FcΔC1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3.FcΔC1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml -0.3mg/ml) of either component were injected onto a Pharmacia Superose 12 PC 3.2/30 column
25 equilibrated in 6M guanidinium chloride and eluted with the same

solution at flow rate 40μl/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak area of the components was 1.16. The molar

5   ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak height of the components was 1.10.

Example 27: Determination of the Stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion

10 **Chromatography with On-Line Light Scattering**

Complex preparation

VEGF165 was mixed with CHO transiently expressed

15 Flt1D2.Flk1D3.FcΔC1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.FcΔC1(a)) and incubated overnight at 4°C.

(a) Size Exclusion Chromatography (SEC) with On-Line Light Scattering

20

Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex.

25 Samples were injected onto a Superose 12 HR 10/30 column

79

(Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was

5   calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390

10   (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165.

15   Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

20   **Example 28:   Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)**

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic

25   fragments were analyzed and identified by HPLC coupled with mass

80

spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify

5   fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36.

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide

10   bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined

15   whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

20   There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is

observed on Asn65 and Asn120.   Sites of glycosylation are highlighted

by underline in the Figure 36.

Example 29:   Pharmacokinetic Analysis of Modified Flt

5   **Receptors**

(a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)

10   Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of

Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a),

CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently

expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6,

24hrs, 2 days, 3 days and 6 days after injection.   The sera were assayed

15   in an ELISA designed to detect Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a).   The ELISA involves

coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc

(A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting

with an anti-Fc antibody linked to horse radish peroxidase.   The results

20   of this experiments are shown in Figure 37.   The T_{max} for Flt1(1-3)-Fc

(A40) was at 6 hrs while the T_{max} for the transient and stable

Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was

24hrs.   The C_{max} for Flt1(1-3)-Fc (A40) was 8μg/ml.   For both

transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max}

was 18µg/ml and the C_{max} for the stable VEGFR1R2-FcΔC1(a) was 30µg/ml.

(b) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40),

5 ## Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a)

Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice

10 were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting

15 with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas , Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more. The results of this experiment are shown in Figure 38.

20

Example 30: Evaluation of the Ability of

Flt1D2.Flk1D3.FcΔC1(a) to Inhibit Tumor Growth In Vivo

To evaluate the ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit tumor

25 growth in vivo a model in which tumor cell suspensions are implanted

subcutaneously on the right flank of male severe combined

immunodeficiency (SCID) mice was employed. Two cell lines, the

human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121)

and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of

5   which exhibit distinctly different morphologies and growth

characteristics, were used in the assay. The first dose of

Flt1D2.Flk1D3.FcΔC1(a) (at 25mg/Kg or as indicated in Figures 39 and

40) was given on the day of tumor implantation. Animals subsequently

received subcutaneous injections of Flt1(1-3)-Fc (A40),

10   Flt1D2.Flk1D3.FcΔC1(a) or vehicle either every other day (EOD) or two

times per week (2X/wk) for a period of 2 weeks. After 2 weeks,

animals were perfused with fixative, tumors were removed and

samples were blinded. Tumor volume was determined by measuring the

length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc

15   (A40) and Flt1D2.Flk1D3.FcΔC1(a) significantly reduced the growth of

tumors formed by HT-1080 and C6 cells. The results of these

experiments are shown in Figure 39 and Figure 40.

Example 31:   The Effect of VEGF165 and Modified Flt

20   **Receptors in Female Reproductive System**

The stereotypic pattern of vascular remodeling which occur in the

uterus and ovary over the course of the reproductive cycle has been

well characterized, making these tissues particularly well suited to

25   the study of mechanisms which regulate angiogenesis, vascular

84

remodeling and vascular regression. Indeed, *in situ* hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates (Phillips et al,

5   1990; Ravindranath et al, 1992; Shweiki et al, 1993; Kamat et al, 1995). As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these

10   organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-angiogenic factors, most prominently VEGF.

15   For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al, 1995; Shifren et al, 1996; Guidi et al, 1996; Donnez et al, 1998). Overexpression of VEGF is also thought to play a pathogenic role in the

20   establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome (McClure et al, 1994; Levin et al, 1998) and preeclampsia (Baker et al, 1995; Sharkey et al, 1996). In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other

25   tumors (Senger et al, 1983; Boocock et al, 1995). Agents which effectively neutralize the biological actions of VEGF can reasonably be

anticipated to be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks of blastocyst
5   implantation and placental development (Findlay, 1986). VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the
10   maternal and embryonic components of the placental vasculature (Shweiki et al, 1993; Cullinan-Bove and Koos, 1993; Chakraborty et al, 1995; Das et al, 1997). VEGF is also required for luteal angiogenesis and associated progesterone secretion necessary to prepare the uterus for implantation (Ferrara et al, 1998). Thus, agents which inhibit the
15   biological actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

20   While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1 is also expressed by trophoblasts in the placenta in both humans and animals (Clark et al, 1996; He et al, 1999) where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are
25   expressed by choriocarcinoma cell line BeWo (Charnock-Jones et al, 1994), and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and

metastatic ovarian carcinomas not only to express high levels of VEGF, but - in addition to the vascular endothelium - the tumor cells themselves express KDR and/ or Flt1 (Boocock et al, 1995). These findings suggest that VEGF may not only be critically involved in the

5   generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of

10   reproductive origin.

Methods and Results

(a) Assessment of VEGF-Induced Uterine Hyperpermeability

15

Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction

20   results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). In this in vivo model, the normal weight of the rat uterus is about 50 mg and this can be induced to 300-

25   350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at

1hr. after PMSG injection results in about a 50% inhibition of the

increase in uterine wet weight.   Increasing the dose of modified Flt

receptor does not further reduce the increase in wet weight suggesting

5   that there is a VEGF-independent component to this model.   The results

of this experiment are shown in Figure 41.

(a) Assessment of corpus luteum angiogenesis using progesterone as a readout

10

Pregnant mare's serum gonadotrophin (PMSG) is injected

subcutaneously (5 IU) to induce ovulation in prepubertal female rats.

This results in a fully functioning corpus luteum containing a dense

network of blood vessels after 4 days that allows for the secretion of

15   progesterone into the blood stream in order to prepare the uterus for

implantation.   The induction of angiogenesis in the corpus luteum

requires VEGF; therefore, blocking VEGF would result in a lack of new

blood vessels and thus a lack of progesterone secreted into the blood

stream.   In this in vivo model, resting levels of progesterone are about

20   5ng/ml and this can be induced to a level of 25-40ng/ml after PMSG.

Subcutaneous injection of Flt1(1-3)-Fc (A40) or

Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG

injection results in a complete inhibition of the progesterone induction

on day 4.   The results of this experiment are shown in Figure 42A-42B.

25

Example 33: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and

5   tested in balb/c mice for their pharmacokinetic profile. Both PEGylated

forms of Flt1(1-3)-Fc were found to have much better PK profiles than

Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the

PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

Example 34: VEGF165 ELISA to Test Affinity of Modified Flt1

10   ## Receptor Variants

10pM of VEGF165 was incubated overnight at room temperature with

modified Flt1 receptor variants ranging from 160pM to 0.1pM. The

15   modified Flt1 receptor variants used in this experiment were Flt1(1-

3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed

Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-

FcΔC1(a), Flt1-(1-3$_{NAS}$)-Fc, Flt1(1-3$_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3

$_{NAS}$)-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic

20   amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting

in the incorporation of two new glycosylation sites and a net reduction

of five positive charges, both with the purpose of reducing the

unfavorable effects of this sequence on PK. Flt1(1-3 $_{R->C}$)-Fc is a

modification in which a single arginine (R) residue within the same

25   basic amino acid sequence is changed to a cysteine (C) (KNK<u>R</u>ASVRRR ->

KNK<u>C</u>ASVRRR) to allow for pegylation at that residue, which could then

shield the basic region from exerting its unfavorable effects on PK.

After incubation the solution was transferred to a plate containing a

capture antibody for VEGF165 (R&D). The amount of free VEGF165 was

5   then determined using an antibody to report free VEGF165. This showed

that the modified Flt1 receptor variant with the highest affinity for

VEGF165 (determined as the lowest amount of free VEGF165) was

Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc

(A40) and then by Flt1(1-3$_{R->C}$)-Fc, Flt1(1-3$_{NAS}$)-Fc and

10   Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

WE CLAIM:

1.   An isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising:

5   (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to

            (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and wherein the nucleotide

10   sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

15

2.   The isolated nucleic acid of claim 1 wherein the first VEGF receptor is Flt1.

3.   The isolated nucleic acid of claim 1 wherein the second VEGF receptor is Flk1.

20   receptor is Flk1.

4.   The isolated nucleic acid of claim 1 wherein the second VEGF receptor is Flt4.

25

5.   The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

5

6.   The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

10

7.   The isolated nucleic acid molecule of claim 1, wherein the multimerizing component comprises an immunoglobulin domain.

8.   The isolated nucleic acid molecule of claim 1, wherein the immunoglobulin

15   domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

9.   An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic

20   acid molecule consists essentially of a nucleotide sequence selected from the group consisting of:

        (a)   the nucleotide sequence set forth in Figure 21A-21C
        (b)   the nucleotide sequence set forth in Figure 22A-22C;
        (c)   the nucleotide sequence set forth in Figure 24A-24C; and

25   (d)   a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b) or (c) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

30 10.   An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic

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acid molecule consists essentially of a nucleotide sequence selected from the group consisting of:

(a)   the nucleotide sequence set forth in Figure 13A-13D;

(b)   the nucleotide sequence set forth in Figure 14A-14C;

5   (c)   the nucleotide sequence set forth in Figure 15A-15C;

(d)   the nucleotide sequence set forth in Figure 16A-16D; and

(e)   a nucleotide sequence which as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c) or (d) and which encodes a fusion polypeptide molecule having the biological activity of the modified

10   Flt1 receptor fusion polypeptide.

11.   A fusion polypeptide encoded by the isolated nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10.

15   12.   A composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide of claim 10.

13.   The composition of claim 12, wherein the multimer is a dimer.

20   14.   The composition of claim 13 and a carrier.

15. ·   A vector which comprises the nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10.

25   16.   An expression vector comprising a nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10 wherein the nucleic acid molecule is operatively linked to an expression control sequence.

17.   A host-vector system for the production of a fusion polypeptide which

30   comprises the expression vector of claim 16, in a suitable host cell.

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18.   The host-vector system of claim 17, wherein the suitable host cell is a
bacterial cell, yeast cell, insect cell, or mammalian cell.

19.   The host-vector system of claim 17, wherein the suitable host cell is E. coli.

5

20.   The host-vector system of claim 17, wherein the suitable host cell is a COS
cell or a CHO cell.

21.   A method of producing a fusion polypeptide which comprises growing cells
10   of the host-vector system of claim 17, under conditions permitting production of the
fusion polypeptide and recovering the fusion polypeptide so produced.

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22. A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D, *Figure 21A-21C, Figure 22A-22C,* or Figure 24A-24C, which has been modified by acetylation or pegylation.

5   23. The fusion polypeptide of claim 22 wherein the modification is acetylation.

24. The fusion polypeptide of claim 22 wherein the modification is pegylation.

10

25. The fusion polypeptide of claim 23 wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent.

15   26. The fusion polypeptide of claim 23 wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess.

27. The fusion polypeptide of claim 24 wherein the pegylation is 10K

20   or 20K PEG.

28. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 11.

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29.   The method of claim 28, wherein the mammal is a human.

30.   The method of claim 29, wherein the fusion polypeptide is acetylated.

5

31.   The method of claim 29, wherein the fusion polypeptide is pegylated.

                                                          11
32.   The fusion polypeptide of claims 10 which specifically binds the

10   VEGF receptor ligand VEGF.

33.   A method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide of claim
     11
     10.

15

34.   A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the
                                    11
fusion polypeptide of claim 10.

20   35.   The method of claim 34, wherein the mammal is a human.

36.   The method of claim 34, used to attenuate or prevent tumor growth in a human.

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37. The method of claim 34, used to attenuate or prevent edema in a
human.

38. The method of claim 34, used to attenuate or prevent ascites
5   formation in a human.

39. The method of claim 37, wherein the edema is brain edema.

40. The method of claim 38, wherein the ascites is ovarian cancer
10   associated ascites.

41. A fusion polypeptide capable of binding a VEGF polypeptide
comprising:

(a a VEGF receptor component operatively linked to

15   (b) a multimerizing component,

wherein the VEGF receptor component is the only VEGF receptor
component in the fusion polypeptide and consists essentially of the
amino acid sequence of Ig domain 2 of the extracellular domain of a
first VEGF receptor and the amino acid sequence of Ig domain 3 of the
20   extracellular domain of a second VEGF receptor.

42. The fusion polypeptide of claim 41 wherein the first VEGF receptor
is Flt1.

25

43.   The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flk1.

44.   The fusion polypeptide of claim 41 wherein the second VEGF receptor is
5   Flt4.

45.   The fusion polypeptide claim 41, wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF
10   receptor.

46.   The fusion polypeptide of claim 41, wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF
15   receptor.

47.   The fusion polypeptide of claim 41, wherein the multimerizing component comprises an immunoglobulin domain.

20   48.   The fusion polypeptide of claim 41, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

49.   A fusion polypeptide consisting essentially of an amino acid sequence of a
25   modified Flt1 receptor, wherein the amino acid sequence is selected from the group consisting of:

        (a)   the amino acid sequence set forth in Figure 21A-21C
        (b)   the amino acid sequence set forth in Figure 22A-22C; and
        (c)   the amino acid sequence set forth in Figure 24A-24C.

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50.   A fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence is selected from the group consisting of:

(a)   the amino acid sequence set forth in Figure 13A-13D;

(b)   the amino acid sequence set forth in Figure 14A-14C;

5   (c)   the amino acid sequence set forth in Figure 15A-15C; and

(d)   the amino acid sequence set forth in Figure 16A-16D;

51.   A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 41, 42, 43, 44,

10   49 or 50.

52.   A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim 41, 42, 43, 44, 49 or 50.
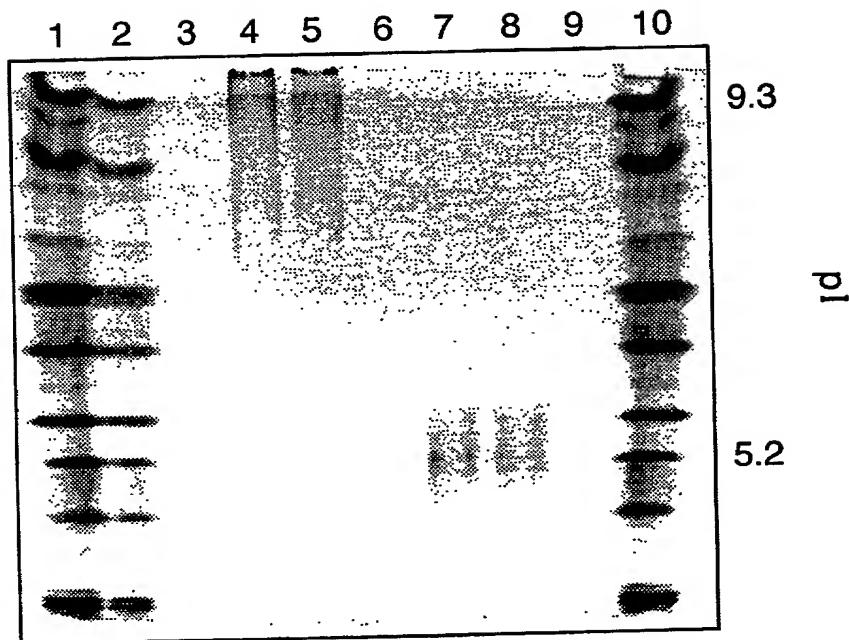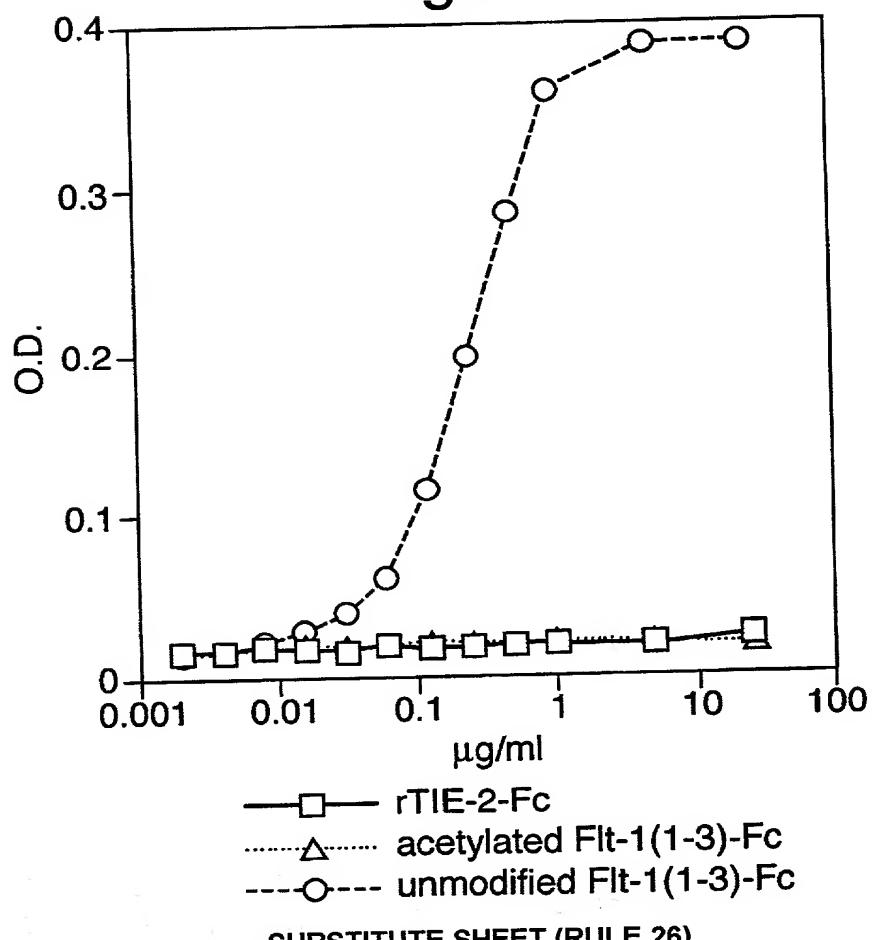
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Fig.1.

Fig.2.

rTIE-2-Fc

acetylated Flt-1(1-3)-Fc

unmodified Flt-1(1-3)-Fc

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Fig.3.

Fig.4.

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Fig.5.

	Unmodified Flt-1(1-3)-Fc
	Unmodified Flt-1(1-3)-Fc + heparin
	Acetylated Flt-1(1-3)-Fc
	Pegylated Flt-1(1-3)-Fc
	rTIE-2-Fc

Days

μg/ml

Fig.6A.

Fig.6B.

Fig.7.

Fig.8.

I←--- Degree of step-acetylation of Flt-1(1-3)-Fc protein ---→I

Legend: 0.5 µg/ml   1.0 µg/ml   5.0 µg/ml

RU (y-axis: -20, 80, 180, 280, 380, 480, 580)

x-axis: 0 10 20 30 40 50 60 70 80 90 100
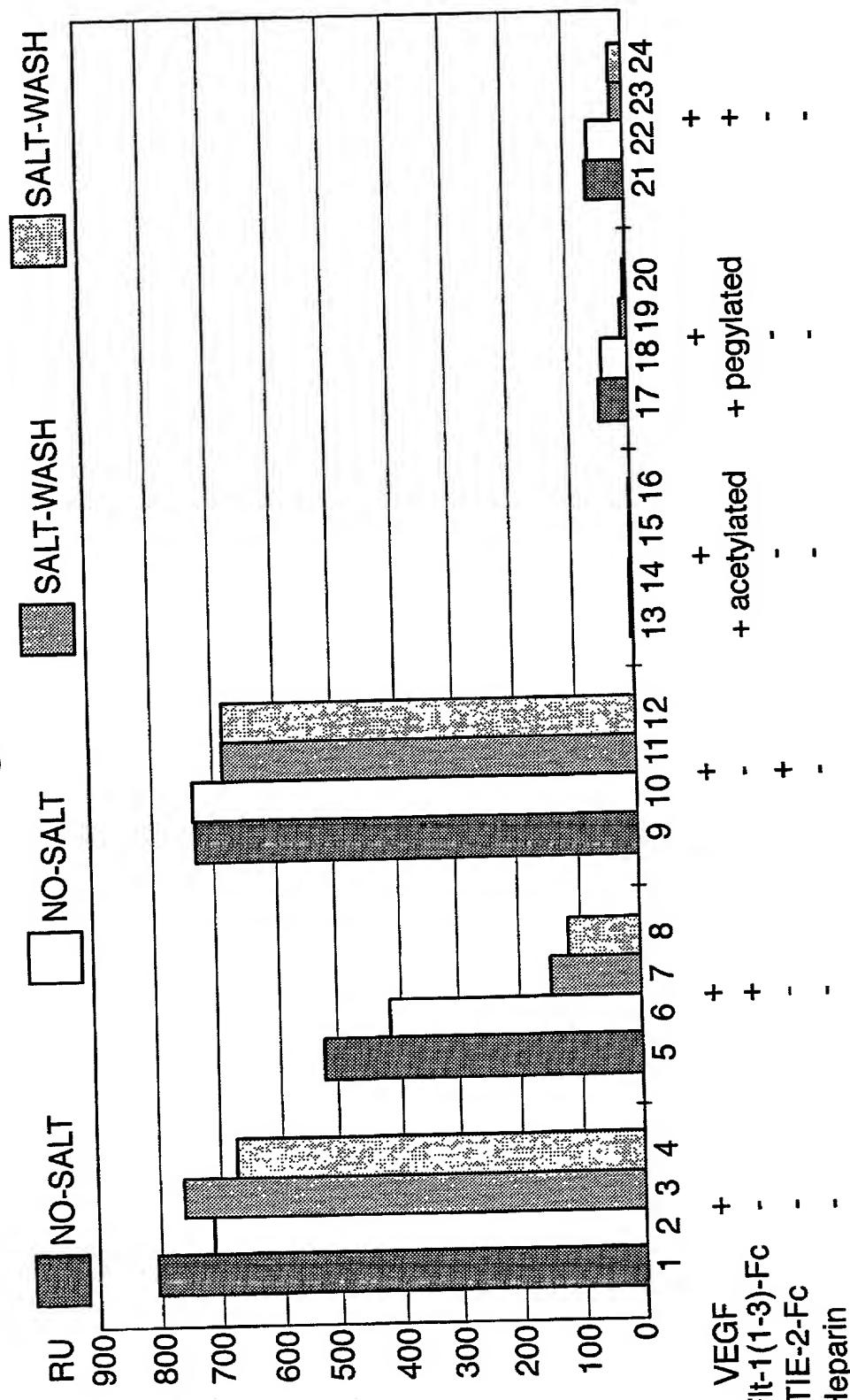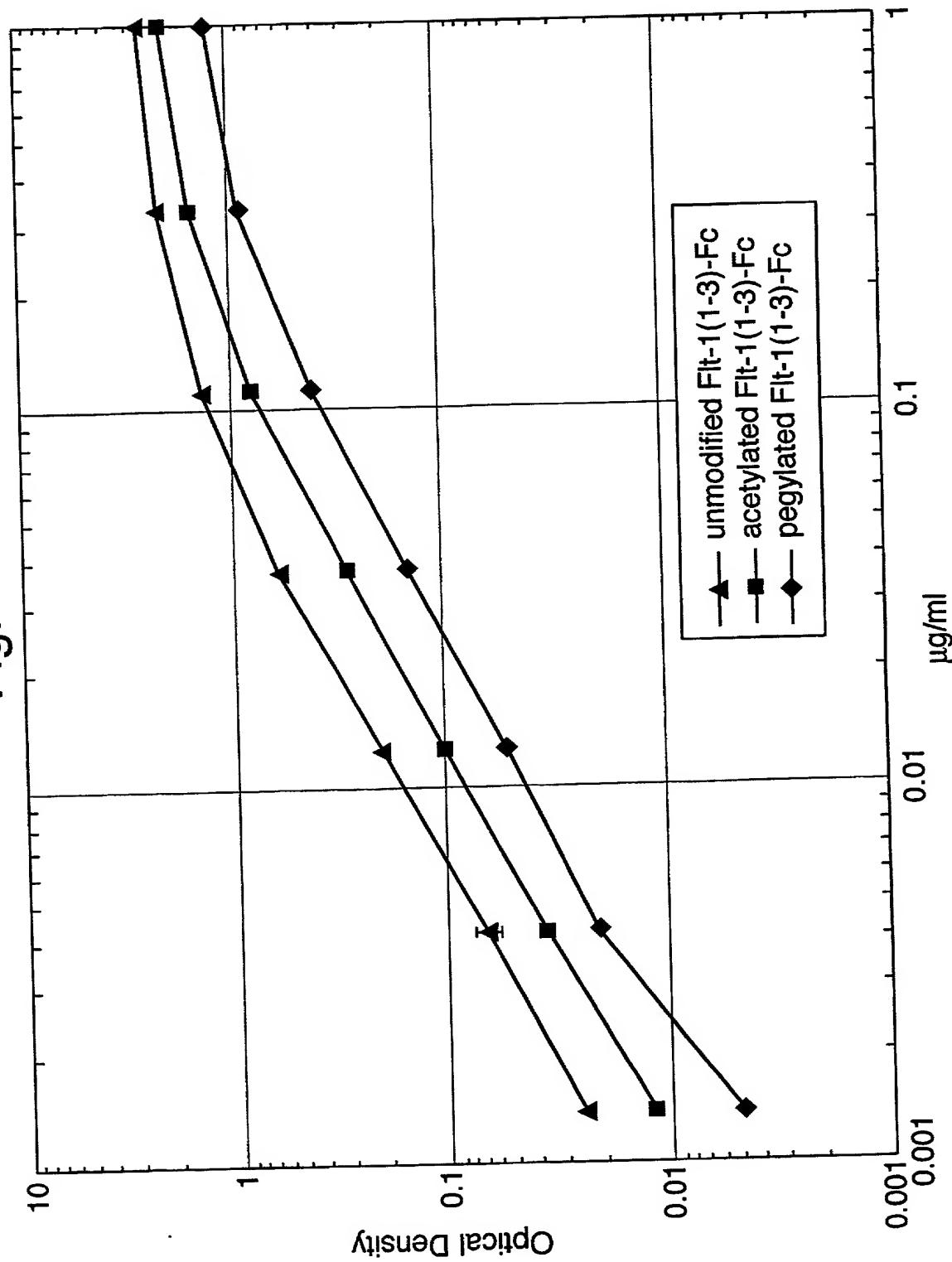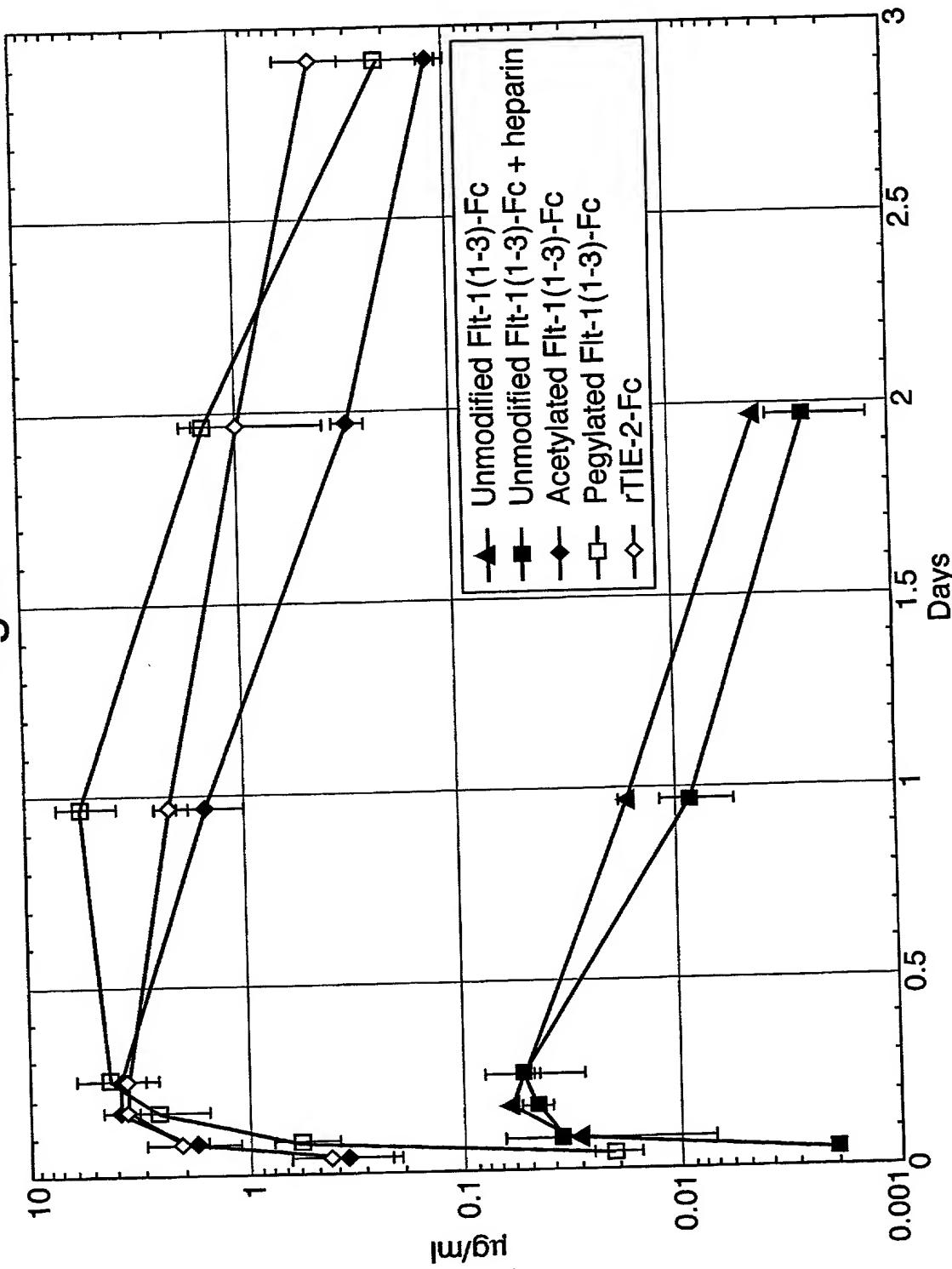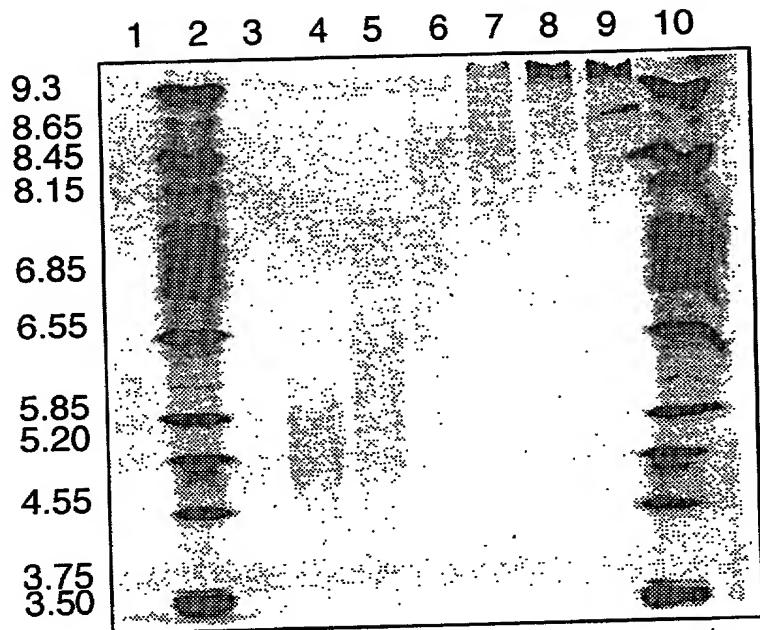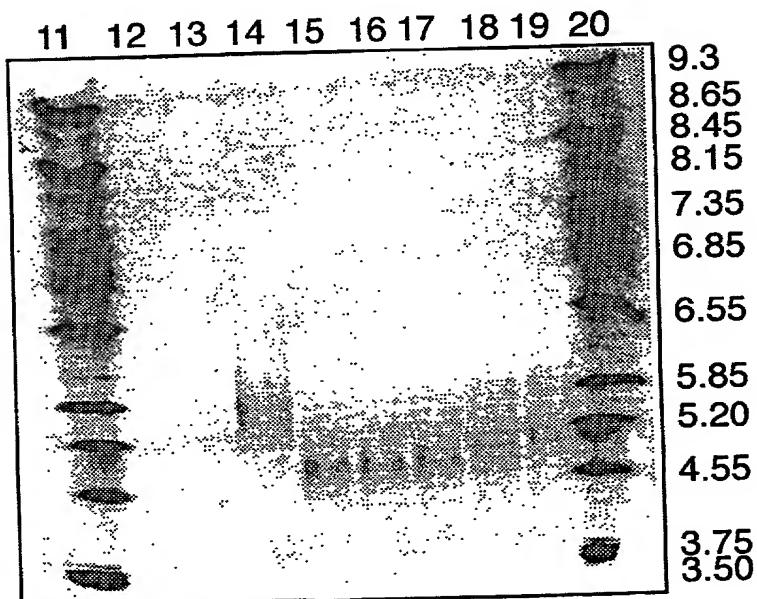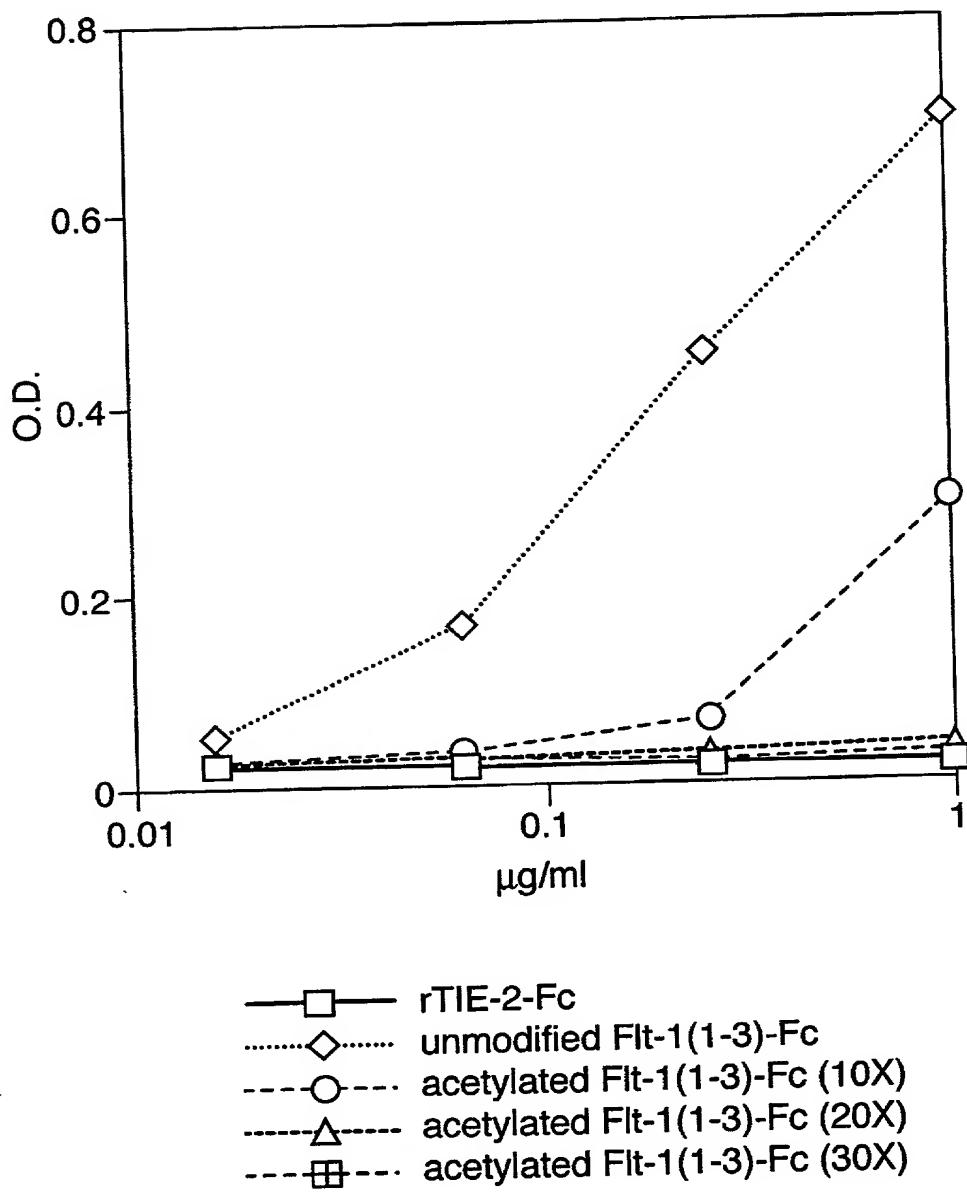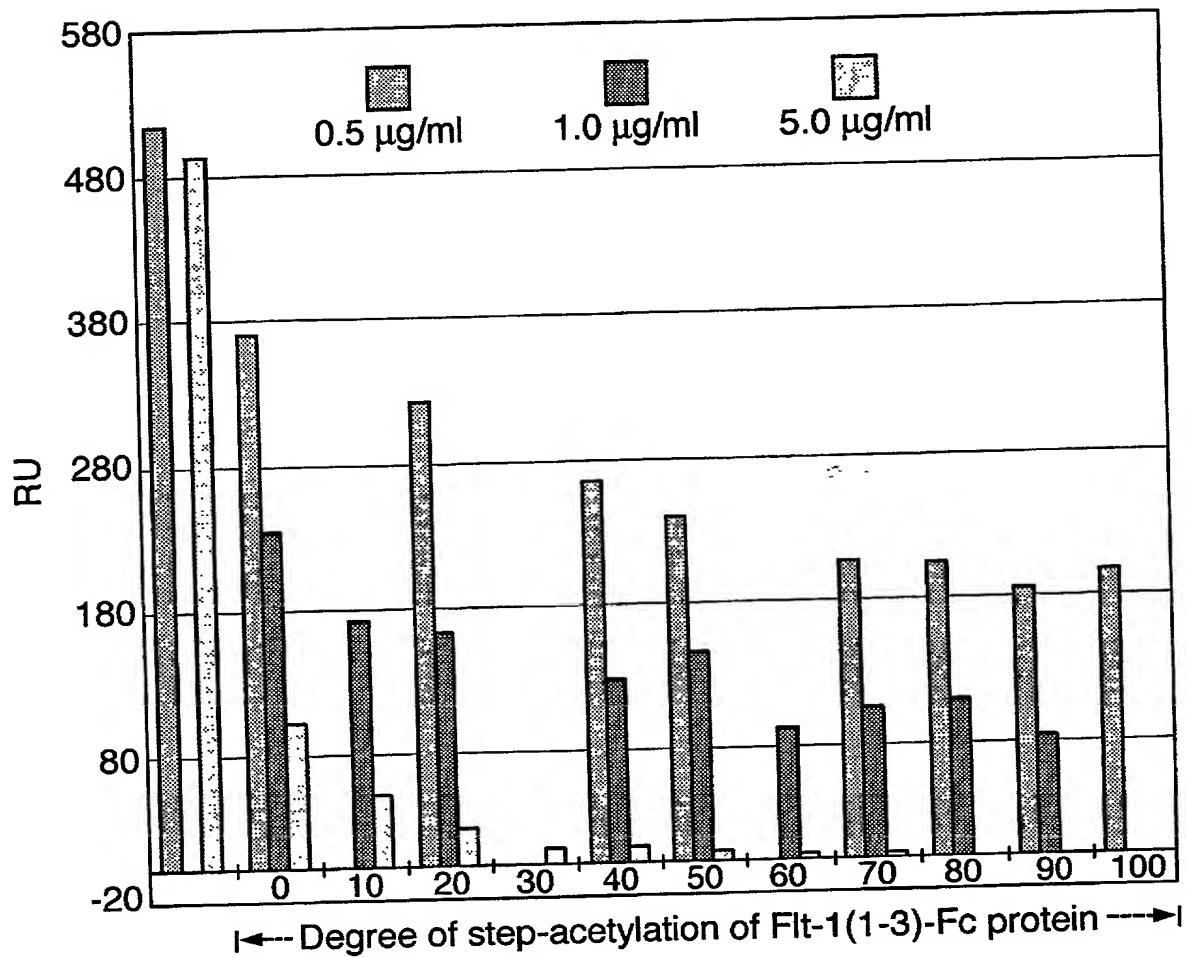
VEGF 0.2 µg/ml	+	+	+	+	+	+	+	+	+	+	+	+
Unmodified Flt-1(1-3)-Fc	-	+	-	-	-	-	-	-	-	-	-	-
Acetylated Flt-1(1-3)-Fc	-	-	+	+	+	+	+	+	+	+	+	+

Fig.9.

Legend:
- ▲ unmodified Flt-1(1-3)-Fc
- ■ acetylated Flt-1(1-3)-Fc (10X)
- ◆ acetylated Flt-1(1-3)-Fc (20X)
- □ acetylated Flt-1(1-3)-Fc (40X)
- ◇ acetylated Flt-1(1-3)-Fc (60X)
- △ acetylated Flt-1(1-3)-Fc (100X)

X-axis: Days
Y-axis: μg/ml

Fig.10A.

```
            10            20            30            40            50            60
         *     *       *     *       *     *       *     *       *     *       *     *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

            70            80            90           100           110           120
         *     *       *     *       *     *       *     *       *     *       *     *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

           130           140           150           160           170           180
         *     *       *     *       *     *       *     *       *     *       *     *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

           190           200           210           220           230           240
         *     *       *     *       *     *       *     *       *     *       *     *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

           250           260           270           280           290           300
         *     *       *     *       *     *       *     *       *     *       *     *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

           310           320           330           340           350           360
         *     *       *     *       *     *       *     *       *     *       *     *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

           370           380           390           400           410           420
         *     *       *     *       *     *       *     *       *     *       *     *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

           430           440           450           460           470           480
         *     *       *     *       *     *       *     *       *     *       *     *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

           490           500           510           520           530           540
         *     *       *     *       *     *       *     *       *     *       *     *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

Fig.10B.

```
         550          560          570          580          590          600
          *       *    *       *    *       *    *       *    *       *    *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>


         610          620          630          640          650          660
          *       *    *       *    *       *    *       *    *       *    *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>


         670          680          690          700          710          720
          *       *    *       *    *       *    *       *    *       *    *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>


         730          740          750          760          770          780
          *       *    *       *    *       *    *       *    *       *    *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>


         790          800          810          820          830          840
          *       *    *       *    *       *    *       *    *       *    *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg>


         850          860          870          880          890          900
          *       *    *       *    *       *    *       *    *       *    *
CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT
Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>


         910          920          930          940          950          960
          *       *    *       *    *       *    *       *    *       *    *
ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA
TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT
Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>


         970          980          990         1000         1010         1020
          *       *    *       *    *       *    *       *    *       *    *
TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT
AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>


        1030         1040         1050         1060         1070         1080
          *       *    *       *    *       *    *       *    *       *    *
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>
```

Fig.10C.

```
          1090           1100           1110           1120           1130           1140
           *      *      *      *      *      *      *      *      *      *      *      *
    TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
    AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT
    Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr>

          1150           1160           1170           1180           1190           1200
           *      *      *      *      *      *      *      *      *      *      *      *
    TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC
    ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG
    Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp>

          1210           1220           1230           1240           1250           1260
           *      *      *      *      *      *      *      *      *      *      *      *
    GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
    CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG
    Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>

          1270           1280           1290           1300           1310           1320
           *      *      *      *      *      *      *      *      *      *      *      *
    CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG
    GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC
    Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys>

          1330           1340           1350           1360           1370           1380
           *      *      *      *      *      *      *      *      *      *      *      *
    TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA
    ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT
    Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>

          1390           1400           1410           1420           1430           1440
           *      *      *      *      *      *      *      *      *      *      *      *
    GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
    CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC
    Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>

          1450           1460           1470           1480           1490           1500
           *      *      *      *      *      *      *      *      *      *      *      *
    AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
    TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC
    Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>

          1510           1520           1530           1540           1550           1560
           *      *      *      *      *      *      *      *      *      *      *      *
    TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC
    ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG
    Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>

          1570           1580           1590           1600           1610           1620
           *      *      *      *      *      *      *      *      *      *      *      *
    GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
    CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC
    Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>
```

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Fig.10D.

```
        1630         1640         1650         1660         1670         1680
     *        *    *        *    *        *    *        *    *        *    *        *
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser>

        1690         1700
     *        *    *        *
CTC TCC CTG TCT CCG GGT AAA TGA
GAG AGG GAC AGA GGC CCA TTT ACT
Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.11.

Fig.12A.

Fig.12B.

Fig.13A.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

          70          80          90         100         110         120
           *           *           *           *           *           *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

         130         140         150         160         170         180
           *           *           *           *           *           *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

         190         200         210         220         230         240
           *           *           *           *           *           *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

         250         260         270         280         290         300
           *           *           *           *           *           *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

         310         320         330         340         350         360
           *           *           *           *           *           *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

         370         380         390         400         410         420
           *           *           *           *           *           *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

         430         440         450         460         470         480
           *           *           *           *           *           *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

         490         500         510         520         530         540
           *           *           *           *           *           *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

SUBSTITUTE SHEET (RULE 26)

Fig.13B.

```
                550         560         570         580         590         600
           *      *      *      *      *      *      *      *      *      *      *      *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

                610         620         630         640         650         660
           *      *      *      *      *      *      *      *      *      *      *      *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

                670         680         690         700         710         720
           *      *      *      *      *      *      *      *      *      *      *      *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

                730         740         750         760         770         780
           *      *      *      *      *      *      *      *      *      *      *      *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

                790         800         810         820         830         840
           *      *      *      *      *      *      *      *      *      *      *      *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG TA CGG TTG
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn>

                850         860         870         880         890         900
           *      *      *      *      *      *      *      *      *      *      *      *
ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT
TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA
Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr>

                910         920         930         940         950         960
           *      *      *      *      *      *      *      *      *      *      *      *
TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT
ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA
Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp>

                970         980         990         1000        1010        1020
           *      *      *      *      *      *      *      *      *      *      *      *
AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA
TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro>

                1030        1040        1050        1060        1070        1080
           *      *      *      *      *      *      *      *      *      *      *      *
GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC
CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr>
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Fig.13C.

```
         1090        1100        1110        1120        1130        1140
          *           *     *      *      *      *     *      *     *      *     *
CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC
GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp>


         1150        1160        1170        1180        1190        1200
          *     *      *      *      *      *      *      *     *      *     *      *
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG
GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC
Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys>


         1210        1220        1230        1240        1250        1260
          *     *      *      *      *      *     *      *     *      *     *      *
CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC
GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His>


         1270        1280        1290        1300        1310        1320
          *     *      *      *     *      *      *     *      *      *     *      *
CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC
GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala>


         1330        1340        1350        1360        1370        1380
          *     *      *      *      *      *      *     *      *      *     *      *
CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC
GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG
Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr>


         1390        1400        1410        1420        1430        1440
          *     *      *      *      *      *      *     *      *      *     *      *
CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA
GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT
Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys>


         1450        1460        1470        1480        1490        1500
          *     *      *      *      *      *      *     *      *      *     *      *
GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC
CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn>


         1510        1520        1530        1540        1550        1560
          *     *      *      *      *      *      *     *      *      *     *      *
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC
ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu>


         1570        1580        1590        1600        1610        1620
          *     *      *      *      *      *      *     *      *      *     *      *
ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG
TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu>
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Fig.13D.

```
              1630          1640          1650          1660          1670
        *       *      *       *      *      *      *       *      *      *
GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.14A.

```
            10           20           30           40           50           60
       *        *        *        *        *        *        *        *        *        *        *        *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

            70           80           90          100          110          120
       *        *        *        *        *        *        *        *        *        *        *        *
ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT
TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA
Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile>

           130          140          150          160          170          180
       *        *        *        *        *        *        *        *        *        *        *        *
ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC
TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TTG TAG
Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile>

           190          200          210          220          230          240
       *        *        *        *        *        *        *        *        *        *        *        *
ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC
TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG
Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile>

           250          260          270          280          290          300
       *        *        *        *        *        *        *        *        *        *        *        *
TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GGG CTT CTG
ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT CCC GAA GAC
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu>

           310          320          330          340          350          360
       *        *        *        *        *        *        *        *        *        *        *        *
ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA
TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT GTA GCT GTT
Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln>

           370          380          390          400          410          420
       *        *        *        *        *        *        *        *        *        *        *        *
ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC
TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG
Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly>

           430          440          450          460          470          480
       *        *        *        *        *        *        *        *        *        *        *        *
CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC
GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC TCT CAA GTT TAC TGG
His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr>

           490          500          510          520          530          540
       *        *        *        *        *        *        *        *        *        *        *        *
TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT
ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val>
```

Fig.14B.

```
        550          560          570          580          590          600
         *       *    *       *    *       *    *       *    *       *    *    *
CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT
GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA
Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser>

        610          620          630          640          650          660
         *       *    *       *    *       *    *       *    *       *    *    *
GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC
CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG
Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly>

        670          680          690          700          710          720
         *       *    *       *    *       *    *       *    *       *    *    *
GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG
CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC
Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu>

        730          740          750          760          770          780
         *       *    *       *    *       *    *       *    *       *    *    *
GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg>

        790          800          810          820          830          840
         *       *    *       *    *       *    *       *    *       *    *    *
ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC
TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe>

        850          860          870          880          890          900
         *       *    *       *    *       *    *       *    *       *    *    *
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG
TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln>

        910          920          930          940          950          960
         *       *    *       *    *       *    *       *    *       *    *    *
TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA
Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>

        970          980          990         1000         1010         1020
         *       *    *       *    *       *    *       *    *       *    *    *
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>

       1030         1040         1050         1060         1070         1080
         *       *    *       *    *       *    *       *    *       *    *    *
ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG
TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>
```

Fig.14C.

```
            1090        1100        1110        1120        1130        1140
      *       *    *       *    *       *    *       *    *       *    *       *
    GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC
    CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG
    Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser>

            1150        1160        1170        1180        1190        1200
      *       *    *       *    *       *    *       *    *       *    *       *
    GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT
    CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA
    Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro>

            1210        1220        1230        1240        1250        1260
      *       *    *       *    *       *    *       *    *       *    *       *
    CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC
    GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG
    Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>

            1270        1280        1290        1300        1310        1320
      *       *    *       *    *       *    *       *    *       *    *       *
    AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC
    TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG
    Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His>

            1330        1340        1350
      *       *    *       *    *       *
    TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
    ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
    Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.15A.

```
         10          20          30          40          50          60
          *           *           *           *           *           *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

         70          80          90         100         110         120
          *           *           *           *           *           *
ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT
TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA
Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile>

        130         140         150         160         170         180
          *           *           *           *           *           *
ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC
TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TTG TAG
Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile>

        190         200         210         220         230         240
          *           *           *           *           *           *
ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC
TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG
Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile>

        250         260         270         280         290         300
          *           *           *           *           *           *
TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GGG CTT CTG
ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT CCC GAA GAC
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu>

        310         320         330         340         350         360
          *           *           *           *           *           *
ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA
TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT GTA GCT GTT
Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln>

        370         380         390         400         410         420
          *           *           *           *           *           *
ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC
TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG
Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly>

        430         440         450         460         470         480
          *           *           *           *           *           *
CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC
GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC TCT CAA GTT TAC TGG
His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr>

        490         500         510         520         530         540
          *           *           *           *           *           *
TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA CGA ATT GAC CAA AGC
ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT GCT TAA CTG GTT TCG
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser>
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SUBSTITUTE SHEET (RULE 26)

Fig.15B.

```
            550         560         570         580         590         600
         *       *   *       *   *       *   *       *   *   *       *   *   *
AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC
TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG
Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp>

            610         620         630         640         650         660
         *       *   *       *   *       *   *       *   *       *   *       *
AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA
TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT
Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser>

            670         680         690         700         710         720
         *       *   *       *   *       *   *       *   *   *       *   *   *
GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr>

            730         740         750         760         770         780
         *       *   *       *   *       *   *       *   *       *   *       *
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA
ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro>

            790         800         810         820         830         840
         *       *   *       *   *       *   *       *   *   *       *   *   *
AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

            850         860         870         880         890         900
         *       *   *       *   *       *   *       *   *       *   *       *
GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT
CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His>

            910         920         930         940         950         960
         *       *   *       *   *       *   *       *   *   *       *   *   *
AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC
TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG
Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val>

            970         980         990        1000        1010        1020
         *       *   *       *   *       *   *       *   *   *       *   *   *
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC
GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn>

           1030        1040        1050        1060        1070        1080
         *       *   *       *   *       *   *       *   *   *       *   *   *
AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>
```

Fig.15C.

```
         1090          1100          1110          1120          1130          1140
          *       *      *       *       *      *       *      *       *      *       *
CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG
GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu>


         1150          1160          1170          1180          1190          1200
          *       *      *       *       *      *       *      *       *      *       *
ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG
TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly>


         1210          1220          1230          1240          1250          1260
          *       *      *       *       *      *       *      *       *      *       *
CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe>


         1270          1280          1290          1300          1310          1320
          *       *      *       *       *      *       *      *       *      *       *
CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC
GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>


         1330          1340          1350          1360          1370          1380
          *       *      *       *       *      *       *      *       *      *       *
TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG
AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro>


          *
GGT AAA TGA
CCA TTT ACT
Gly Lys ***>
```

Fig.16A.

```
              10           20           30           40           50           60
               *     *      *     *      *     *      *     *      *     *      *     *
         ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
         TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
         Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

              70           80           90          100          110          120
               *     *      *     *      *     *      *     *      *     *      *     *
         ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
         TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
         Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

             130          140          150          160          170          180
               *     *      *     *      *     *      *     *      *     *      *     *
         CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
         GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
         His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

             190          200          210          220          230          240
               *     *      *     *      *     *      *     *      *     *      *     *
         TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
         ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
         Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

             250          260          270          280          290          300
               *     *      *     *      *     *      *     *      *     *      *     *
         TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
         ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
         Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

             310          320          330          340          350          360
               *     *      *     *      *     *      *     *      *     *      *     *
         CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
         GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
         His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

             370          380          390          400          410          420
               *     *      *     *      *     *      *     *      *     *      *     *
         GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
         CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
         Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

             430          440          450          460          470          480
               *     *      *     *      *     *      *     *      *     *      *     *
         GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
         CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
         Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

             490          500          510          520          530          540
               *     *      *     *      *     *      *     *      *     *      *     *
         ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
         TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
         Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

Fig.16B.

```
          550         560         570         580         590         600
           *      *      *      *      *      *  ·    *      *      *      *      *      *

GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>


          610         620         630         640         650         660
           *      *      *      *      *      *      *      *      *      *      *      *

GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>


          670         680         690         700         710         720
           *      *      *      *      *      *      *      *      *      *      *      *

CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>


          730         740         750         760         770         780
           *      *      *      *  ·   *      *      *      *      *      *      *      *

AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>


          790         800         810         820         830         840
           *      *      *      *      *      *      *      *      *      *      *      *

AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AAC GCT TCC GTA AGG CGA
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TTG CGA AGG CAT TCC GCT
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Asn Ala Ser Val Arg Arg>


          850         860         870         880         890         900
           *      *      *      *      *      *      *      *      *      *      *      *

CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT
Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>


          910         920         930         940         950         960
           *      *      *      *      *      *      *      *      *      *      *      *

ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA
TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT
Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>


          970         980         990        1000        1010        1020
           *      *      *      *      *      *      *      *      *      *      *      *

TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT
AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>


         1030        1040        1050        1060        1070        1080
           *      *      *      *      *      *      *      *      *      *      *      *

GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>
```

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Fig.16C.

```
           1090        1100        1110        1120        1130        1140
       *       *       *       *       *       *       *       *       *       *       *       *
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr>


           1150        1160        1170        1180        1190        1200
       *       *       *       *       *       *       *       *       *       *       *       *
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp>


           1210        1220        1230        1240        1250        1260
       *       *       *       *       *       *       *       *       *       *       *       *
GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>


           1270        1280        1290        1300        1310        1320
       *       *       *       *       *       *       *       *       *       *       *       *
CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG
GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys>


           1330        1340        1350        1360        1370        1380
       *       *       *       *       *       *       *       *       *       *       *       *
TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA
ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>


           1390        1400        1410        1420        1430        1440
       *       *       *       *       *       *       *       *       *       *       *       *
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>


           1450        1460        1470        1480        1490        1500
       *       *       *       *       *       *       *       *       *       *       *       *
AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>


           1510        1520        1530        1540        1550        1560
       *       *       *       *       *       *       *       *       *       *       *       *
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC
ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>


           1570        1580        1590        1600        1610        1620
       *       *       *       *       *       *       *       *       *       *       *       *
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>
```

Fig.16D.

```
            1630          1640          1650          1660          1670          1680
      *       *     *       *       *     *       *     *     *       *       *     *
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser>
```

```
            1690          1700
      *       *     *       *
CTC TCC CTG TCT CCG GGT AAA TGA
GAG AGG GAC AGA GGC CCA TTT ACT
Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.17.

Fig.18.

Fig.19.

Fig.20.

Fig.21A.

                                        >EcoRI_site
                                             |
    10   20   30   40   50 | 60   70   80
AAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCGATCCCCGGGCGAGCTCGAATTCGCAACCACCATGGTCAGCTAC
TTCGAACCCGACGTCCAGCTAGCTGAGATCTCCTAGCTAGGGGCCCGCTCGAGCTTAAGCGTTGGTGGTACCAGTCGATG
                                                            M V S Y>
                                                            1   4
                                                            _____>

                                        >BspEI_bridge
                                             |
    90   100   110   120   130   140 | 150   160
TGGGACACCGGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTCACAGGATCTAGTTCCGGAGGTAGACCTTTCGT
ACCCTGTGGCCCCAGGACGACACGCGCGACGAGTCGACAGACGAAGAGTGTCCTAGATCAAGGCCTCCATCTGGAAAGCA
   W D T G V L L C A L L S C L L L T G S S>
   _____FLT1 SS_____>
                                                            S G>
                                                            _____>
                                                               G R P F V>
                                                               31
                                                               _____>

   170   180   190   200   210   220   230   240
AGAGATGTACAGTGAAATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACGTCAC
TCTCTACATGTCACTTTAGGGGCTTTAATATGTGTACTGACTTCCTTCCCTCGAGCAGTAAGGGACGGCCCAATGCAGTG
   E M Y S E I P E I I H M T E G R E L V I P C R V T S>
                                                                              57
   _____HFLT1 D2_____>

   250   260   270   280   290   300   310   320
CTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACGCATAATCTGGGACAGTAGA
GATTGTAGTGACAATGAAATTTTTTTCAAAGGTGAACTGTGAAACTAGGGACTACCTTTTGCGTATTAGACCCTGTCATCT
   P N I T V T L K K F P L D T L I P D G K R I I W D S R>
                                                                                 84
   _____HFLT1 D2_____>

   330   340   350   360   370   380   390   400
AAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGCTTCTGACCTGTGAAGCAACAGTCAATGGGCATTTGTA
TTCCCGAAGTAGTATAGTTTACGTTGCATGTTTCTTTATCCCGAAGACTGGACACTTCGTTGTCAGTTACCCGTAAACAT
   K G F I I S N A T Y K E I G L L T C E A T V N G H L Y>
                                                                                 111
   _____HFLT1 D2_____>

   410   420   430   440   450   460   470   480
TAAGACAAACTATCTCACACATCGACAAACCAATACAATCATAGATGTGGTTCTGAGTCCGTCTCATGGAATTGAACTAT
ATTCTGTTTGATAGAGTGTGTAGCTGTTTGGTTATGTTAGTATCTACACCAAGACTCAGGCAGAGTACCTTAACTTGATA
   K T N Y L T H R Q T N T I I D>
   _____HFLT1 D2_____>
                                                V V L S P S H G I E L>
                                                                              137
                                                _____HFLK1 D3_____>

SUBSTITUTE SHEET (RULE 26)

Fig.21B.

```
        490       500       510       520       530       540       550       560
CTGTTGGAGAAAAGCTTGTCTTAAATTGTACAGCAAGAACTGAACTAAATGTGGGGATTGACTTCAACTGGGAATACCCT
GACAACCTCTTTTCGAACAGAATTTAACATGTCGTTCTTGACTTGATTTACACCCCTAACTGAAGTTGACCCTTATGGGA
  S  V  G  E  K  L  V  L  N  C  T  A  R  T  E  L  N  V  G  I  D  F  N  W  E  Y  P>
                                                                              164
_____HFLK1 D3_____>
```

```
        570       580       590       600       610       620       630       640
TCTTCGAAGCATCAGCATAAGAAACTTGTAAACCGAGACCTAAAAACCCAGTCTGGGAGTGAGATGAAGAAATTTTTGAG
AGAAGCTTCGTAGTCGTATTCTTTGAACATTTGGCTCTGGATTTTTGGGTCAGACCCTCACTCTACTTCTTTAAAAACTC
  S  S  K  H  Q  H  K  K  L  V  N  R  D  L  K  T  Q  S  G  S  E  M  K  K  F  L  S>
                                                                              191
_____HFLK1 D3_____>
```

```
        650       660       670       680       690       700       710       720
CACCTTAACTATAGATGGTGTAACCCGGAGTGACCAAGGATTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAAGA
GTGGAATTGATATCTACCACATTGGGCCTCACTGGTTCCTAACATGTGGACACGTCGTAGGTCACCCGACTACTGGTTCT
  T  L  T  I  D  G  V  T  R  S  D  Q  G  L  Y  T  C  A  A  S  S  G  L  M  T  K>
                                                                              217
_____HFLK1 D3_____>
```

```
                                >Srf_Bridge_
                                     |
        730       740       750      |  760       770       780       790       800
AGAACAGCACATTTGTCAGGGTCCATGAAAAGGGCCCGGGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA
TCTTGTCGTGTAAACAGTCCCAGGTACTTTTCCCGGGCCCGCTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTT
  K  N  S  T  F  V  R  V  H  E  K>
_____HFLK1 D3_____>
                                 G  P  G>
                                 _____>
                                         D  K  T  H  T  C  P  P  C  P  A  P  E>
                                                                              244
                                 _____FCΔC1(A)_____>
```

```
        810       820       830       840       850       860       870       880
CTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC
GAGGACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTG
  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  T>
                                                                              271
_____FCΔC1(A)_____>
```

```
        890       900       910       920       930       940       950       960
ATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG
TACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC
  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N>
                                                                              297
_____FCΔC1(A)_____>
```

```
        970       980       990      1000      1010      1020      1030      1040
CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGG
GGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACC
  A  K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W>
                                                                              324
_____FCΔC1(A)_____>
```

Fig.21C.

```
      1050      1060      1070      1080      1090      1100      1110      1120
CTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAA
GACTTACCGTTCCTCATGTTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTT
  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P  A  P  I  E  K  T  I  S  K  A  K>
                                                                              351
_____FCΔC1(A)_____>
```

```
                                        >A>C_A_allotype
                                            |
                              >G>T_A_allotype    |
                                     |      |
      1130      1140      1150      1160      1170   |  1180      1190      1200
AGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT
TCCCGTCGGGGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGA
  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T>
                                                                            377
_____FCΔC1(A)_____>
```

```
      1210      1220      1230      1240      1250      1260      1270      1280
GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACC
CGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGG
  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T>
                                                                              404
_____FCΔC1(A)_____>
```

```
                                        >T>C
                                            |
      1290      1300      1310      1320      1330      1340      1350      1360
ACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG
TGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCC
  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G>
                                                                              431
_____FCΔC1(A)_____>
```

```
      1370      1380      1390      1400      1410      1420      1430      1440
GAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA
CTTGCAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCAT
  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G>
                                                                            457
_____FCΔC1(A)_____>
```

```
 >NotI_site
     |
     |1450
AATGAGCGGCCGC
TTACTCGCCGGCG
K   *>
458
_____>
```

Fig.22A.

```
                                              >EcoRI_site
                                                  |
        10        20        30        40        50  |     60        70        80
AAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCGATCCCCGGGCGAGCTCGAATTCGCAACCACCATGGTCAGCTAC
TTCGAACCCGACGTCCAGCTAGCTGAGATCTCCTAGCTAGGGGCCCGCTCGAGCTTAAGCGTTGGTGGTACCAGTCGATG
                                                              M   V   S   Y>
                                                              1               4
                                                              _____>


                                                  >BspEI_bridge
                                                       |
        90       100       110       120       130       140|      150       160
TGGGACACCGGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTCACAGGATCTAGTTCCGGAGGTAGACCTTTCGT
ACCCTGTGGCCCCAGGACGACACGCGCGACGAGTCGACAGACGAAGAGTGTCCTAGATCAAGGCCTCCATCTGGAAAGCA
    W   D   T   G   V   L   L   C   A   L   L   S   C   L   L   L   T   G   S   S>
    _____FLT1 SIGNAL SEQUENCE_____>
                                                              S   G>
                                                              ____>
                                                                  G   R   P   F   V>
                                                                                  31
                                                                  _____>


       170       180       190       200       210       220       230       240
AGAGATGTACAGTGAAATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACGTCAC
TCTCTACATGTCACTTTAGGGGCTTTAATATGTGTACTGACTTCCTTCCCTCGAGCAGTAAGGGACGGCCCAATGCAGTG
     E   M   Y   S   E   I   P   E   I   I   H   M   T   E   G   R   E   L   V   I   P   C   R   V   T   S>
                                                                                                      57
    _____FLT1 IG DOMAIN 2_____>


       250       260       270       280       290       300       310       320
CTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACGCATAATCTGGGACAGTAGA
GATTGTAGTGACAATGAAATTTTTTCAAAGGTGAACTGTGAAACTAGGGACTACCTTTTGCGTATTAGACCCTGTCATCT
 P   N   I   T   V   T   L   K   K   F   P   L   D   T   L   I   P   D   G   K   R   I   I   W   D   S   R>
                                                                                                      84
    _____FLT1 IG DOMAIN 2_____>


       330       340       350       360       370       380       390       400
AAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGCTTCTGACCTGTGAAGCAACAGTCAATGGGCATTTGTA
TTCCCGAAGTAGTATAGTTTACGTTGCATGTTTCTTTATCCCGAAGACTGGACACTTCGTTGTCAGTTACCCGTAAACAT
  K   G   F   I   I   S   N   A   T   Y   K   E   I   G   L   L   T   C   E   A   T   V   N   G   H   L   Y>
                                                                                                      111
    _____FLT1 IG DOMAIN 2_____>


       410       420       430       440       450       460       470       480
TAAGACAAACTATCTCACACATCGACAAACCAATACAATCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGC
ATTCTGTTTGATAGAGTGTGTAGCTGTTTGGTTATGTTAGTATCTATAGGTCGACAACGGGTCCTTCAGCGACCTCGACG
    K   T   N   Y   L   T   H   R   Q   T   N   T   I   I   D>
    _____FLT1 IG DOMAIN 2_____>
                                                I   Q   L   L   P   R   K   S   L   E   L>
                                                                                      137
                                                ____VEGFR3 (FLT4) IG DOMAIN 3____>
```

Fig.22B.

```
        490       500       510       520       530       540       550       560
TGGTAGGGGAGAAGCTGGTCCTCAACTGCACCGTGTGGGCTGAGTTTAACTCAGGTGTCACCTTTGACTGGGACTACCCA
ACCATCCCCTCTTCGACCAGGAGTTGACGTGGCACACCCGACTCAAATTGAGTCCACAGTGGAAACTGACCCTGATGGGT
  L  V  G  E  K  L  V  L  N  C  T  V  W  A  E  F  N  S  G  V  T  F  D  W  D  Y  P>
                                                                              164
_____VEGFR3 (FLT4) IG DOMAIN 3_____>


        570       580       590       600       610       620       630       640
GGGAAGCAGGCAGAGCGGGGTAAGTGGGTGCCCGAGCGACGCTCCCAACAGACCCACACAGAACTCTCCAGCATCCTGAC
CCCTTCGTCCGTCTCGCCCCATTCACCCACGGGCTCGCTGCGAGGGTTGTCTGGGTGTGTCTTGAGAGGTCGTAGGACTG
   G  K  Q  A  E  R  G  K  W  V  P  E  R  R  S  Q  Q  T  H  T  E  L  S  S  I  L  T>
                                                                              191
_____VEGFR3 (FLT4) IG DOMAIN 3_____>


        650       660       670       680       690       700       710       720
CATCCACAACGTCAGCCAGCACGACCTGGGCTCGTATGTGTGCAAGGCCAACAACGGCATCCAGCGATTTCGGGAGAGCA
GTAGGTGTTGCAGTCGGTCGTGCTGGACCCGAGCATACACACGTTCCGGTTGTTGCCGTAGGTCGCTAAAGCCCTCTCGT
  I  H  N  V  S  Q  H  D  L  G  S  Y  V  C  K  A  N  N  G  I  Q  R  F  R  E  S>
                                                                              217
_____VEGFR3 (FLT4) IG DOMAIN 3_____>


        730       740       750       760       770       780       790       800
CCGAGGTCATTGTGCATGAAAATGGCCCGGGCGACAAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGG
GGCTCCAGTAACACGTACTTTTACCGGGCCCGCTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAGGACCCC
  T  E  V  I  V  H  E  N>
___VEGFR3 (FLT4) IG_____>
                     G  P  G>
                     _____>
                          D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G>
                                                                       244
                     _____FCΔC1 - A ALLOTYPE_____>


        810       820       830       840       850       860       870       880
GGACCGTCAGTCTTCCTCTTCCCCCCAAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGT
CCTGGCAGTCAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCA
  G  P  S  V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V>
                                                                              271
_____FCΔC1 - A ALLOTYPE_____>


        890       900       910       920       930       940       950       960
GGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAA
CCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTT
  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T>
                                                                           297
_____FCΔC1 - A ALLOTYPE_____>


        970       980       990       1000      1010      1020      1030      1040
AGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGC
TCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACCGACTTACCG
  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G>
                                                                              324
_____FCΔC1 - A ALLOTYPE_____>
```

Fig.22C.

```
        1050      1060      1070      1080      1090      1100      1110      1120
AAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCC
TTCCTCATGTTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGTCGG
  K   E   Y   K   C   K   V   S   N   K   A   L   P   A   P   I   E   K   T   I   S   K   A   K   G   Q   P>
                                                                                              351
        _____FCΔC1 - A ALLOTYPE_____>
```

```
                                                  >A>C_A_allotype
                                                        |
                                                  >G>T_A_allotype
                                                    |   |
        1130      1140      1150      1160      |1170      1180      1190      1200
CCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA
GGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAGT
    R   E   P   Q   V   Y   T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V>
                                                                                              377
        _____FCΔC1 - A ALLOTYPE_____>
```

```
        1210      1220      1230      1240      1250      1260      1270      1280
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCC
TTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGG
  K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P   E   N   N   Y   K   T   T   P   P>
                                                                                              404
        _____FCΔC1 - A ALLOTYPE_____>
```

```
                                                  >T>C
                                                    |
        1290      1300      1310      1320      1330      1340      1350      1360
GTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTT
CACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCAGAA
  V   L   D   S   D   G   S   F   F   L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G   N   V   F>
                                                                                              431
        _____FCΔC1 - A ALLOTYPE_____>
```

```
                                                                              >NotI_site
                                                                                  |
        1370      1380      1390      1400      1410      1420      1430      1440
CTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGCGG
GAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTACTCGCC
    S   C   S   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S   L   S   L   S   P   G   K   *>
                                                                                              455
        _____FCΔC1 - A ALLOTYPE_____>
```

```
CCGC
GGCG
```

Fig.23.

● Flt1D2Flk1D3.FcdeltaC1(a)

△ Flt1D2VEGFR3D3.FcdeltaC1(a)

▽ TIE2-Fc

■ Flt1(1-3)-Fc
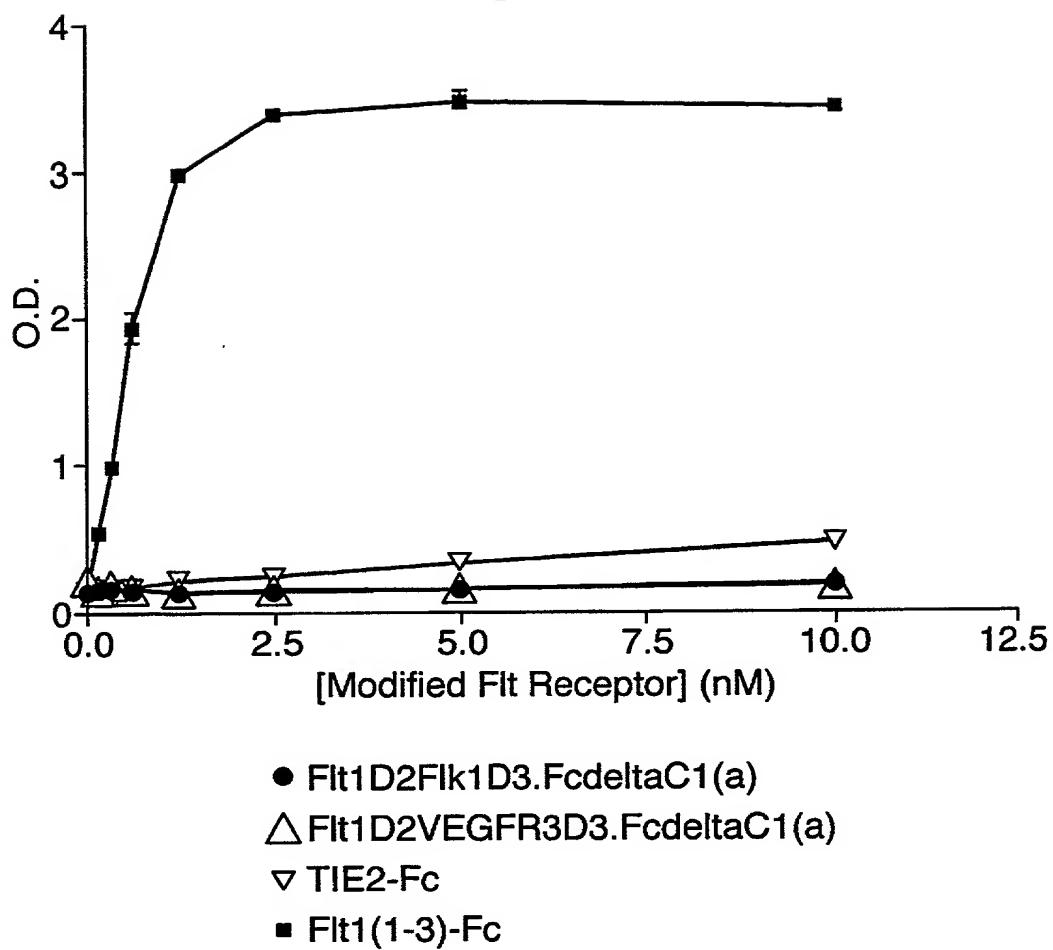
Fig.24A.

```
          10             20             30             40             50             60
           *              *              *              *              *              *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
 M   V   S   Y   W   D   T   G   V   L   L   C   A   L   L   S   C   L   L   L>
 1               5           hFLT1 SIGNAL SEQUENCE        15                    20>


          70             80             90            100            110            120
           *              *              *              *              *              *
ACA GGA TCT AGT TCC GGA AGT GAT ACC GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC
TGT CCT AGA TCA AGG CCT TCA CTA TGG CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG
 T   G   S   S   G>
21_hFLT1 SIGNAL SEQ_26>
                         S   D   T   G   R   P   F   V   E   M   Y   S   E   I>
                        _27          30    hFLT1 IG DOMAIN 2                 40>


         130            140            150            160            170            180
           *              *              *              *              *              *
CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA
GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT
 P   E   I   I   H   M   T   E   G   R   E   L   V   I   P   C   R   V   T   S>
41              45           hFLT1 IG DOMAIN 2        55                    60>


         190            200            210            220            230            240
           *              *              *              *              *              *
CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA
GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT
 P   N   I   T   V   T   L   K   K   F   P   L   D   T   L   I   P   D   G   K>
61              65           hFLT1 IG DOMAIN 2        75                    80>


         250            260            270            280            290            300
           *              *              *              *              *              *
CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA
GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT
 R   I   I   W   D   S   R   K   G   F   I   I   S   N   A   T   Y   K   E   I>
81              85           hFLT1 IG DOMAIN 2        95                    100>


         310            320            330            340            350            360
           *              *              *              *              *              *
GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA
CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT
 G   L   L   T   C   E   A   T   V   N   G   H   L   Y   K   T   N   Y   L   T>
101             105           hFLT1 IG DOMAIN 2        115                   120>


         370            380            390            400            410            420
           *              *              *              *              *              *
CAT CGA CAA ACC AAT ACA ATC ATA GAT GTG GTT CTG AGT CCG TCT CAT GGA ATT GAA CTA
GTA GCT GTT TGG TTA TGT TAG TAT CTA CAC CAA GAC TCA GGC AGA GTA CCT TAA CTT GAT
 H   R   Q   T   N   T   I   I   D>
121         hFLT1 IG DOMAIN 2      129_>
                         V   V   L   S   P   S   H   G   I   E   L>
                        130          hFLK1 IG DOMAIN 3            140>
```

10/009852

Fig.24B.

```
        430         440         450         460         470         480
         *           *           *           *           *           *
TCT GTT GGA GAA AAG CTT GTC TTA AAT TGT ACA GCA AGA ACT GAA CTA AAT GTG GGG ATT
AGA CAA CCT CTT TTC GAA CAG AAT TTA ACA TGT CGT TCT TGA CTT GAT TTA CAC CCC TAA
 S   V   G   E   K   L   V   L   N   C   T   A   R   T   E   L   N   V   G   I>
141_____145_____hFLK1 IG DOMAIN 3_____155_____160>


        490         500         510         520         530         540
         *           *           *           *           *           *
GAC TTC AAC TGG GAA TAC CCT TCT TCG AAG CAT CAG CAT AAG AAA CTT GTA AAC CGA GAC
CTG AAG TTG ACC CTT ATG GGA AGA AGC TTC GTA GTC GTA TTC TTT GAA CAT TTG GCT CTG
 D   F   N   W   E   Y   P   S   S   K   H   Q   H   K   K   L   V   N   R   D>
161_____165_____hFLK1 IG DOMAIN 3_____175_____180>


        550         560         570         580         590         600
         *           *           *           *           *           *
CTA AAA ACC CAG TCT GGG AGT GAG ATG AAG AAA TTT TTG AGC ACC TTA ACT ATA GAT GGT
GAT TTT TGG GTC AGA CCC TCA CTC TAC TTC TTT AAA AAC TCG TGG AAT TGA TAT CTA CCA
 L   K   T   Q   S   G   S   E   M   K   K   F   L   S   T   L   T   I   D   G>
181_____185_____hFLK1 IG DOMAIN 3_____195_____200>


        610         620         630         640         650         660
         *           *           *           *           *           *
GTA ACC CGG AGT GAC CAA GGA TTG TAC ACC TGT GCA GCA TCC AGT GGG CTG ATG ACC AAG
CAT TGG GCC TCA CTG GTT CCT AAC ATG TGG ACA CGT CGT AGG TCA CCC GAC TAC TGG TTC
 V   T   R   S   D   Q   G   L   Y   T   C   A   A   S   S   G   L   M   T   K>
201_____205_____hFLK1 IG DOMAIN 3_____215_____220>


        670         680         690         700         710         720
         *           *           *           *           *           *
AAG AAC AGC ACA TTT GTC AGG GTC CAT GAA AAG GAC AAA ACT CAC ACA TGC CCA CCG TGC
TTC TTG TCG TGT AAA CAG TCC CAG GTA CTT TTC CTG TTT TGA GTG TGT ACG GGT GGC ACG
 K   N   S   T   F   V   R   V   H   E   K>
221_____hFLK1 IG DOMAIN 3_____231>
                                              D   K   T   H   T   C   P   P   C>
                                            232_____hFCΔC1 A _____240>


        730         740         750         760         770         780
         *           *           *           *           *           *
CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG
 P   A   P   E   L   L   G   G   P   S   V   F   L   F   P   P   K   P   K   D>
241_____245_____hFCΔC1 A _____255_____260>


        790         800         810         820         830         840
         *           *           *           *           *           *
ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA
TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT
 T   L   M   I   S   R   T   P   E   V   T   C   V   V   V   D   V   S   H   E>
261_____265_____hFCΔC1 A _____275_____280>


        850         860         870         880         890         900
         *           *           *           *           *           *
GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT
 D   P   E   V   K   F   N   W   Y   V   D   G   V   E   V   H   N   A   K   T>
281_____285_____hFCΔC1 A _____295_____300>
```

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Fig.24C.

```
        910          920          930          940          950          960
         *            *            *            *            *            *
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG
TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC
 K   P   R   E   E   Q   Y   N   S   T   Y   R   V   V   S   V   L   T   V   L>
301_____305_____hFCΔC1 A _____315_____320>

        970          980          990         1000         1010         1020
         *            *            *            *            *            *
CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT
 H   Q   D   W   L   N   G   K   E   Y   K   C   K   V   S   N   K   A   L   P>
321_____325_____hFCΔC1 A _____335_____340>

       1030         1040         1050         1060         1070         1080
         *            *            *            *            *            *
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC
CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG
 A   P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y>
341_____345_____hFCΔC1 A _____355_____360>

       1090         1100         1110         1120         1130         1140
         *            *            *            *            *            *
ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG
 T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V>
361_____365_____hFCΔC1 A _____375_____380>

       1150         1160         1170         1180         1190         1200
         *            *            *            *            *            *
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC
TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG
 K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P   E   N>
381_____385_____hFCΔC1 A _____395_____400>

       1210         1220         1230         1240         1250         1260
         *            *            *            *            *            *
AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC
 N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F   L   Y   S   K>
401_____405_____hFCΔC1 A _____415_____420>

       1270         1280         1290         1300         1310         1320
         *            *            *            *            *            *
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT
GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA
 L   T   V   D   K   S   R   W   Q   Q   G   N   V   F   S   C   S   V   M   H>
421_____425_____hFCΔC1 A _____435_____440>

       1330         1340         1350         1360         1370
         *            *            *            *            *
GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
 E   A   L   H   N   H   Y   T   Q   K   S   L   S   L   S   P   G   K   *>
441_____445_____hFCΔC1 A _____455_____458____>
```

Fig.25A.

DME - Challenge

+ Flt1D2VEGFR3D3.FcΔC1(a)

+ Flt1D2Flk1D3.FcΔC1(a)

+ R→C

+ NAS

+ΔB2

+ A40

+ Flt1 (1-3) Fc

VEGF 165

VEGF - R2 →

1.5 Fold Modified Flt1 Receptor

Fig.25B.

DME - Challenge

+ Flt1D2VEGFR3D3.FcΔC1(a)

+ Flt1D2Flk1D3.FcΔC1(a)

+ R→C

+ NAS

+ΔB2

+ A40

+ Flt1 (1-3) Fc

VEGF 165

VEGF - R2 →

3.0 Fold Modified Flt1 Receptor

Fig.25C.

VEGF - R2 →

6.0 Fold Modified
Flt1 Receptor

Column labels (left to right): VEGF 165, + Flt1 (1-3) Fc, + A40, + ΔB2, + NAS, + R→C, + Flt1D2Flk1D3.FcΔC1(a), + Flt1D2VEGFR3D3.FcΔC1(a), DME - Challenge

Fig.26A.

DME - Challenge
tCHO-VEGFR1R2.FcΔC1(a)
sCHO-Flt1D2Flk1D3.FcΔC1(a) ⎫ 2.0 Fold Modified Flt1 Receptor
tCHO-Flt1D2Flk1D3.FcΔC1(a)
VEGF 165
tCHO-VEGFR1R2.FcΔC1(a)
sCHO-Flt1D2Flk1D3.FcΔC1(a) ⎫ 1.0 Fold Modified Flt1 Receptor
tCHO-Flt1D2Flk1D3.FcΔC1(a)

VEGF - R2 →

VEGF - R2 →

Fig.26B.

DME - Challenge
tCHO-VEGFR1R2.FcΔC1(a)
sCHO-Flt1D2Flk1D3.FcΔC1(a) ⎫ 4.0 Fold Modified Flt1 Receptor
tCHO-Flt1D2Flk1D3.FcΔC1(a)
VEGF 165
tCHO-VEGFR1R2.FcΔC1(a)
sCHO-Flt1D2Flk1D3.FcΔC1(a) ⎫ 3.0 Fold Modified Flt1 Receptor
tCHO-Flt1D2Flk1D3.FcΔC1(a)

Fig.27.

- —○— Flt1D2Flk1D3.FcdeltaC1(a)
- —■— Flt1D2VEGFR3D3.FcdeltaC1(a)
- —◆— Flt1 (1-3)-Fc
- —▬— Tie2-Fc

Absorbance 450/570nm

[pM]

Fig.28.

Binding Stoichiometry of hVEGF165 to Flt1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)		
hVEGF165 (nM)	VEGF/Flt1D2Flk1D3.FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)
1	0.93	0.98
10	0.97	0.94
50	1	0.99
Average ± StDev	0.96 ± 0.03	0.97 ± 0.02

Fig.29.
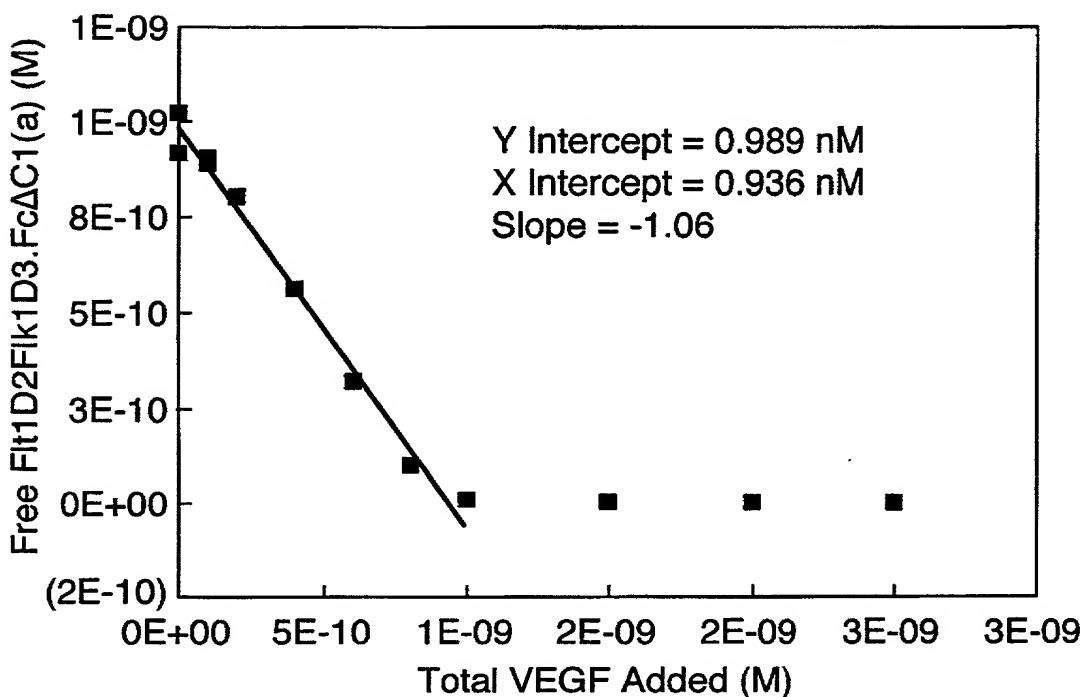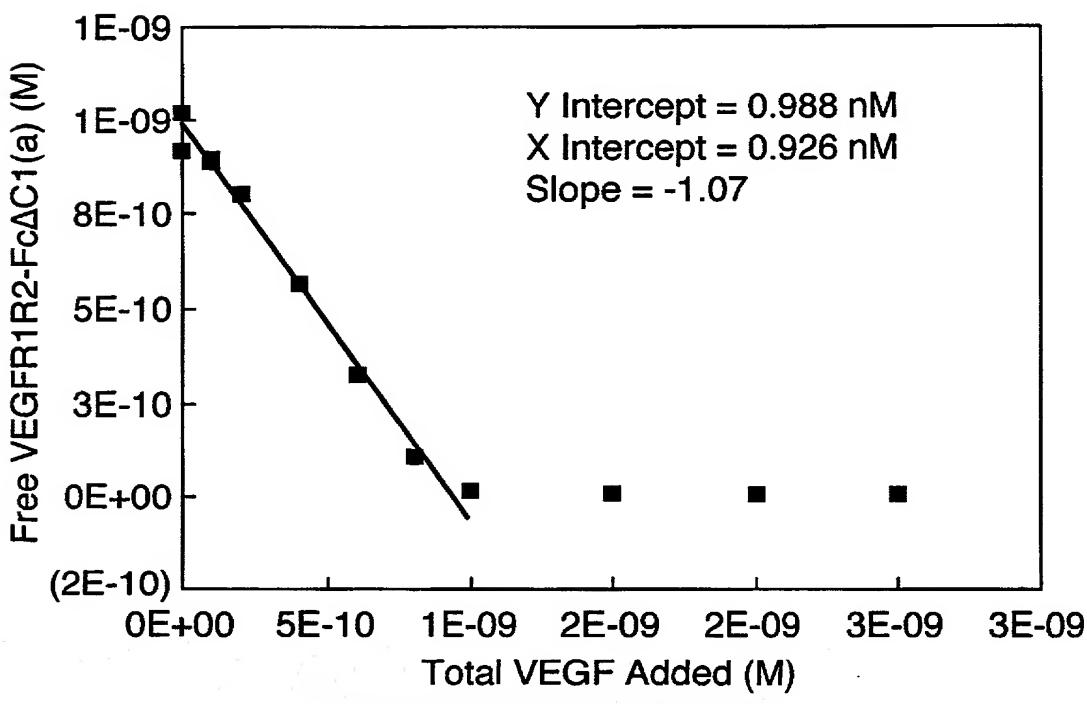
Y Intercept = 0.989 nM
X Intercept = 0.936 nM
Slope = -1.06

Fig.30.

Y Intercept = 0.988 nM
X Intercept = 0.926 nM
Slope = -1.07

Fig.31.
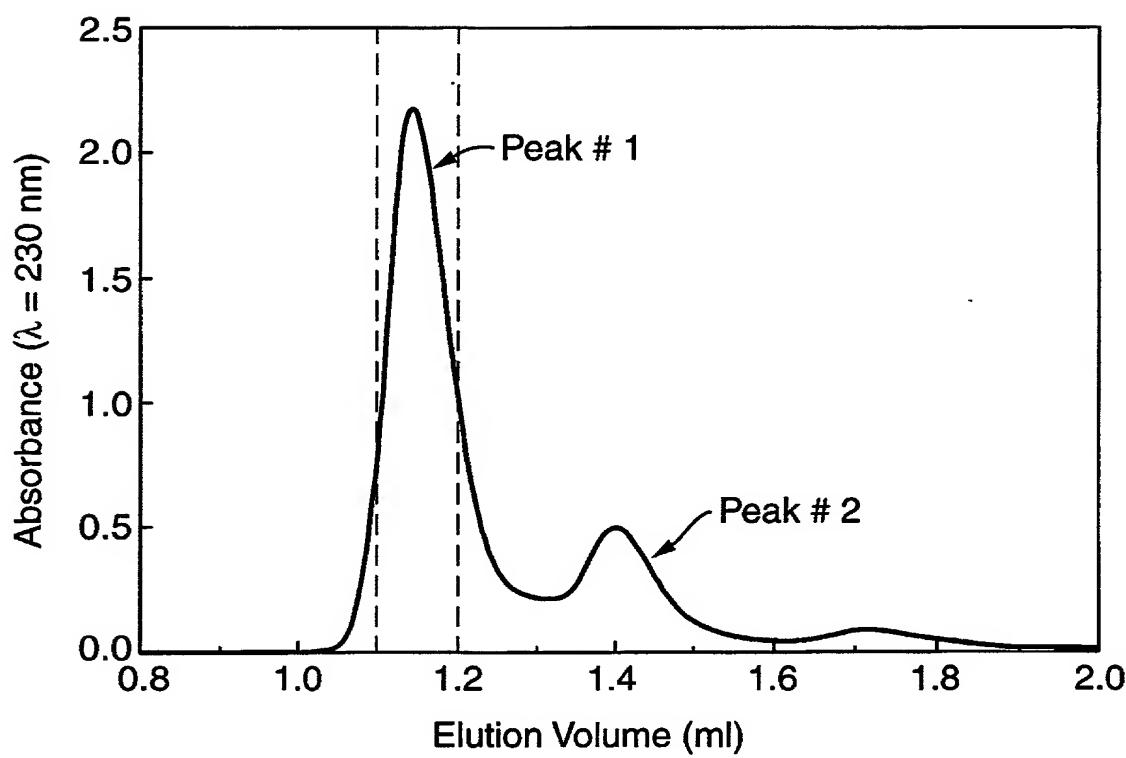
Fig.32.
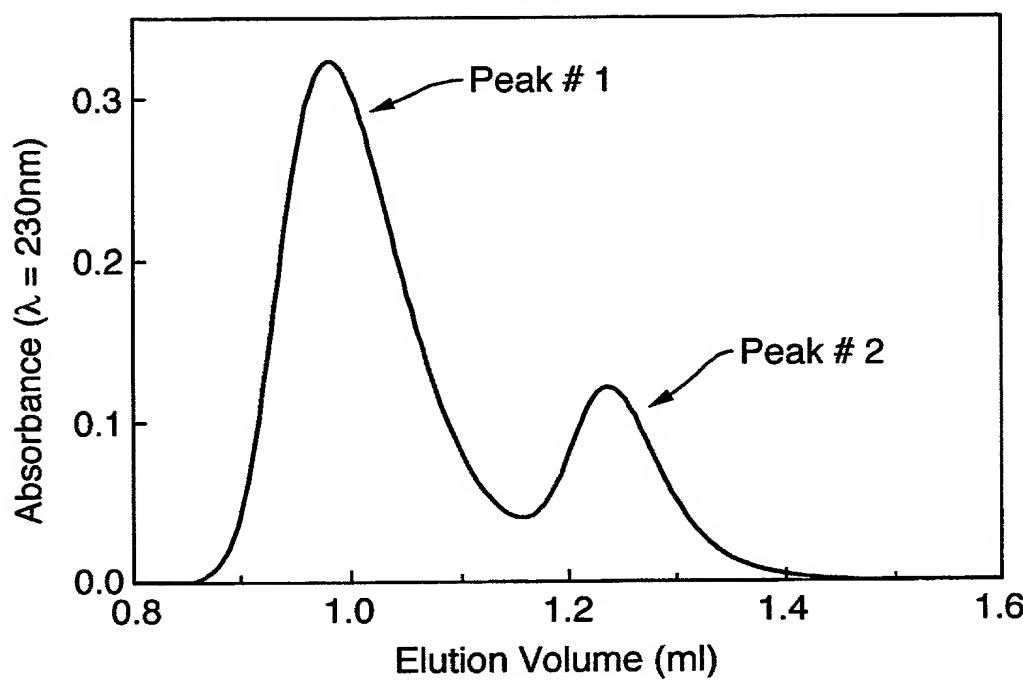
Fig.33.
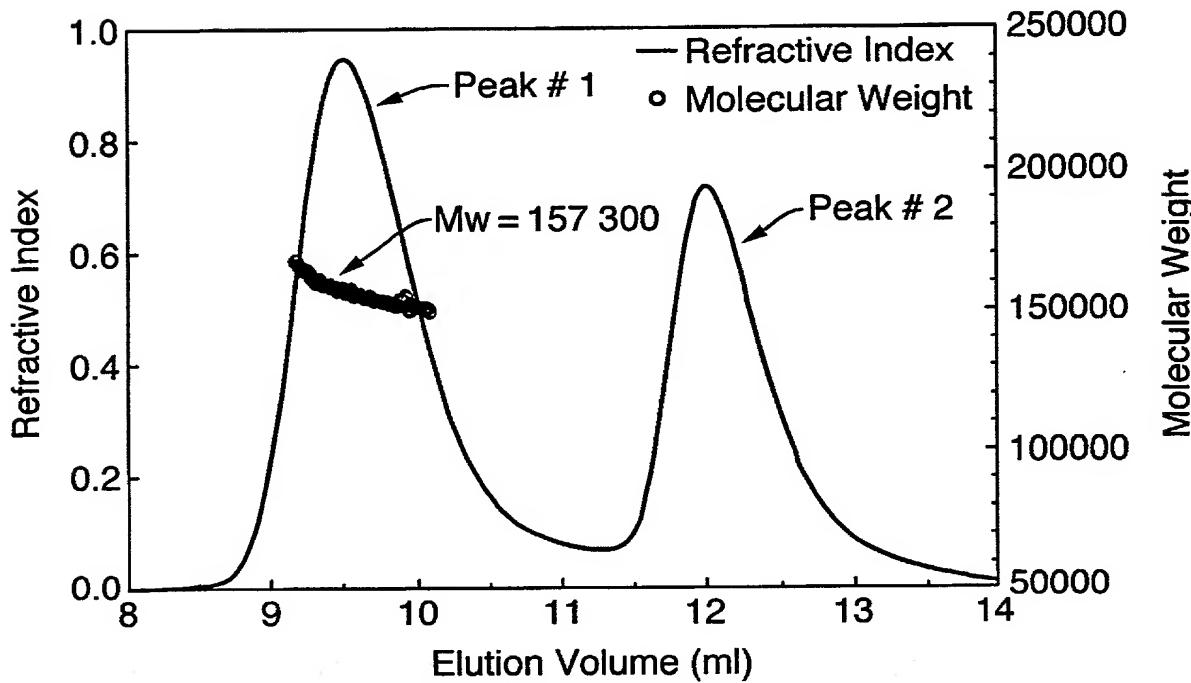
Fig.34.
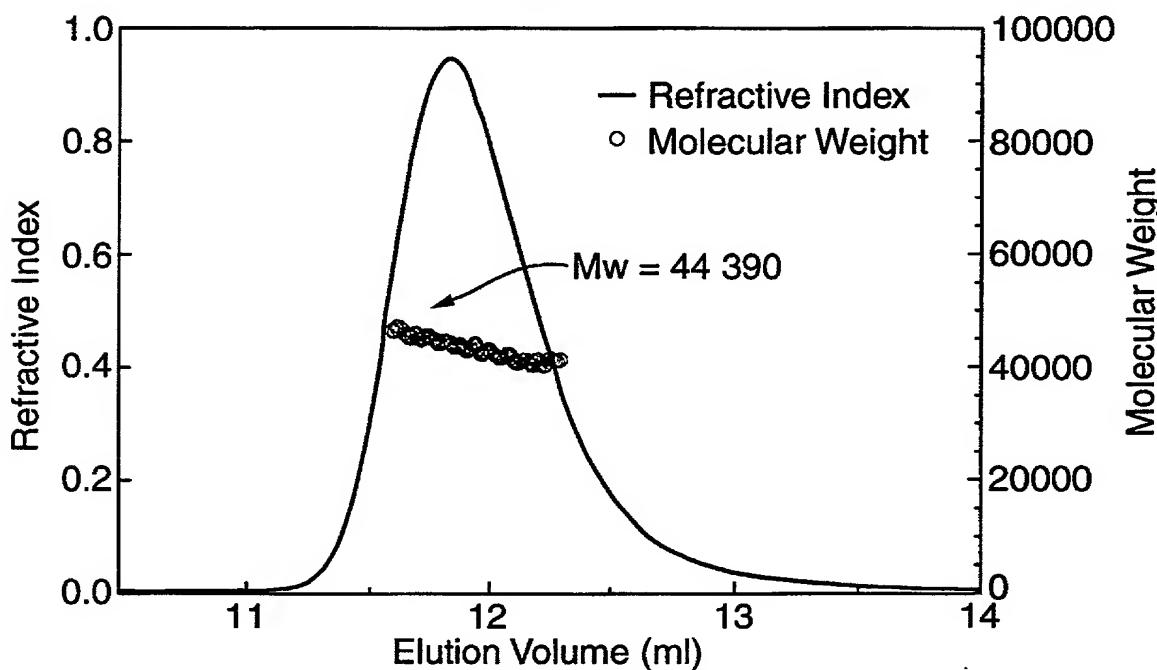
Fig.35.
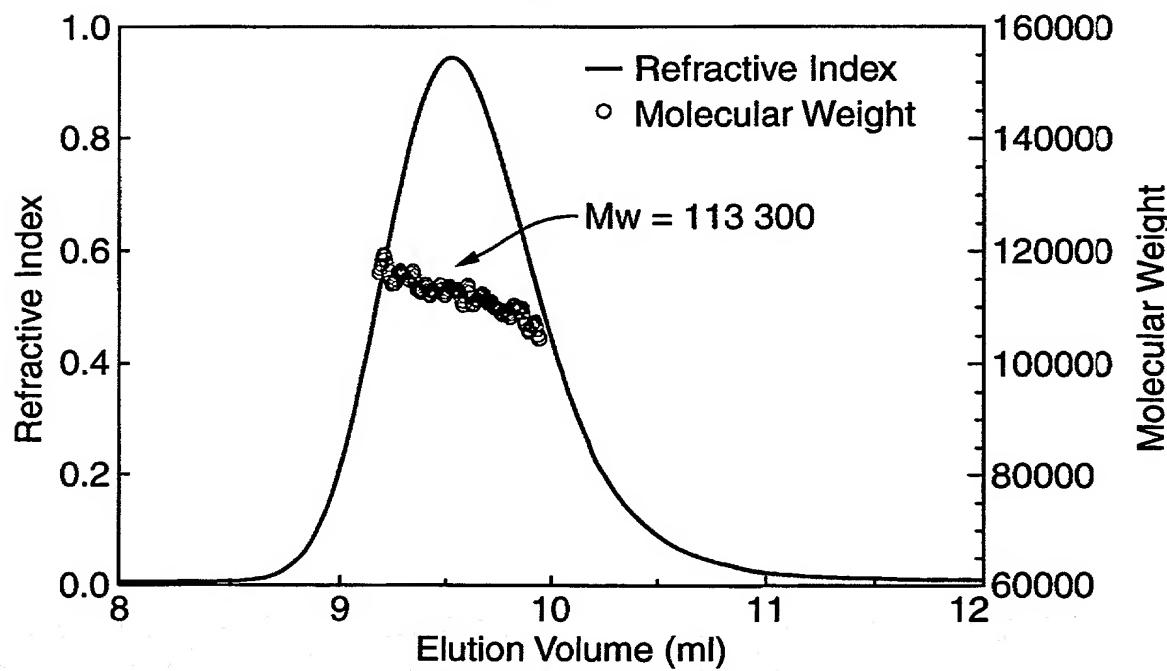
Fig.36.

GRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDG
50

KRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIID
100

VVLSPSHGIELSVGEKLVLNCTARTELNVGDFNWEYPSSKHQHKKLVNR
150
200

DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH
250

EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
300

GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
350

TCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKS
400

RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fig.37.

Fig.38.

Fig.39.

Fig.40.

10/009852
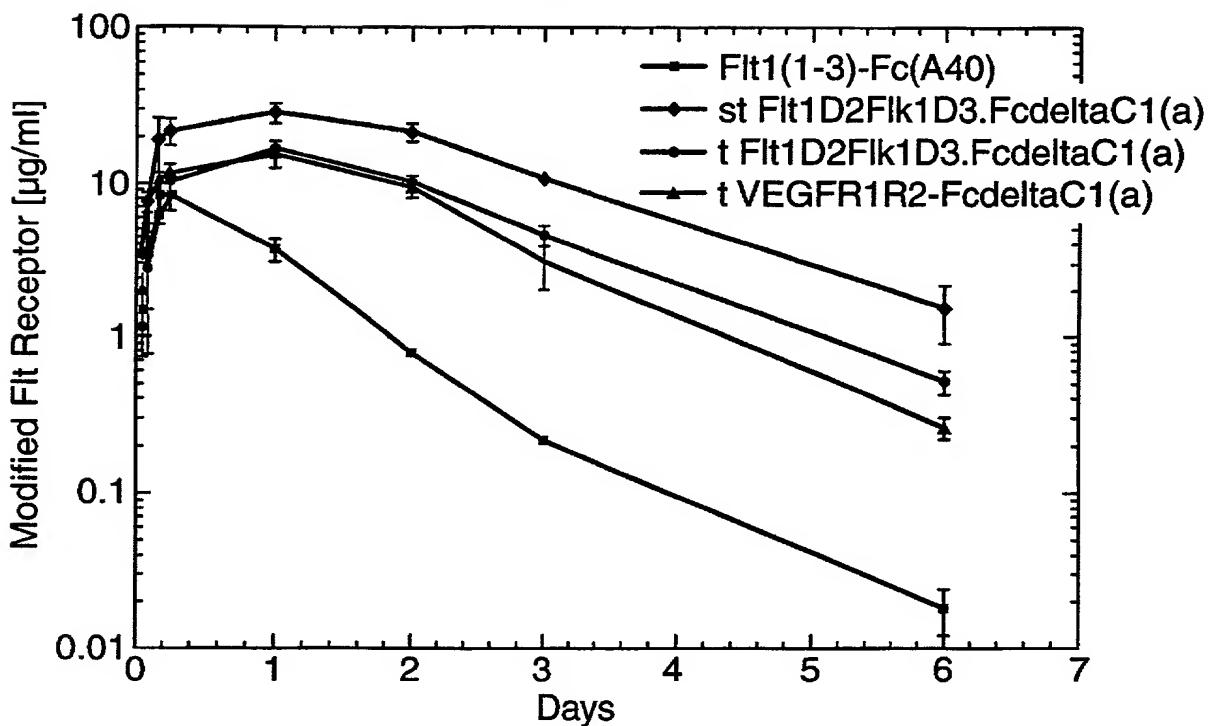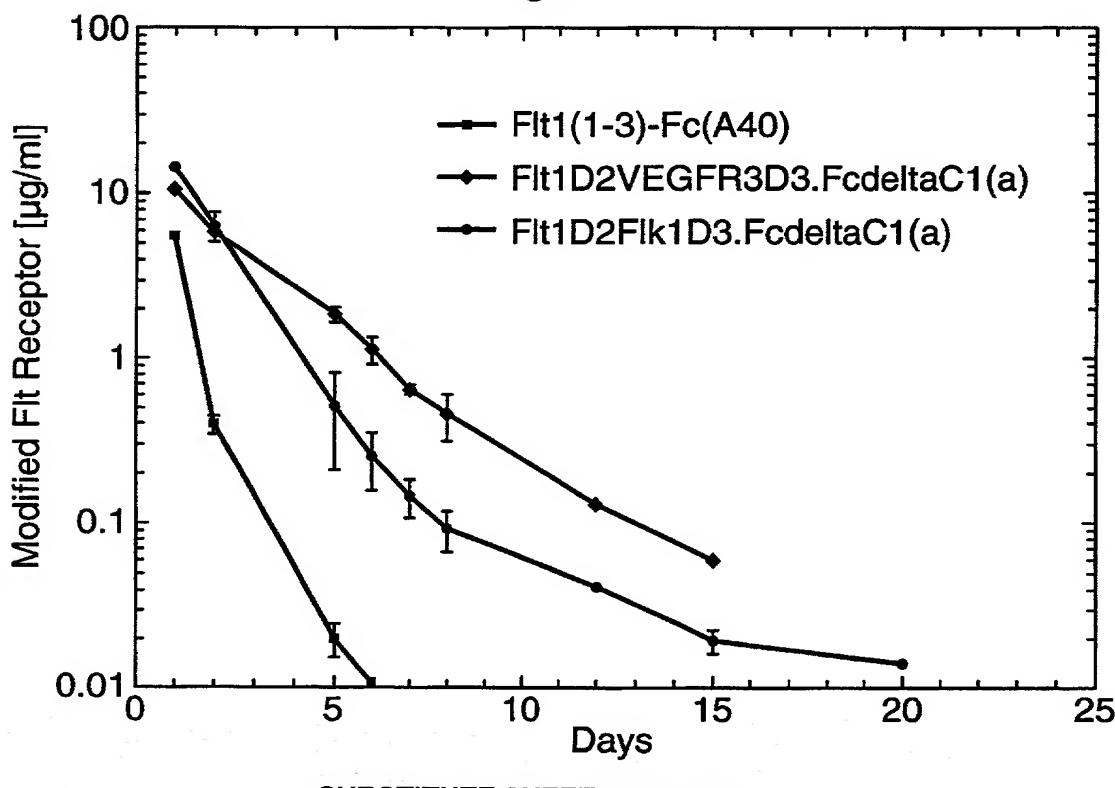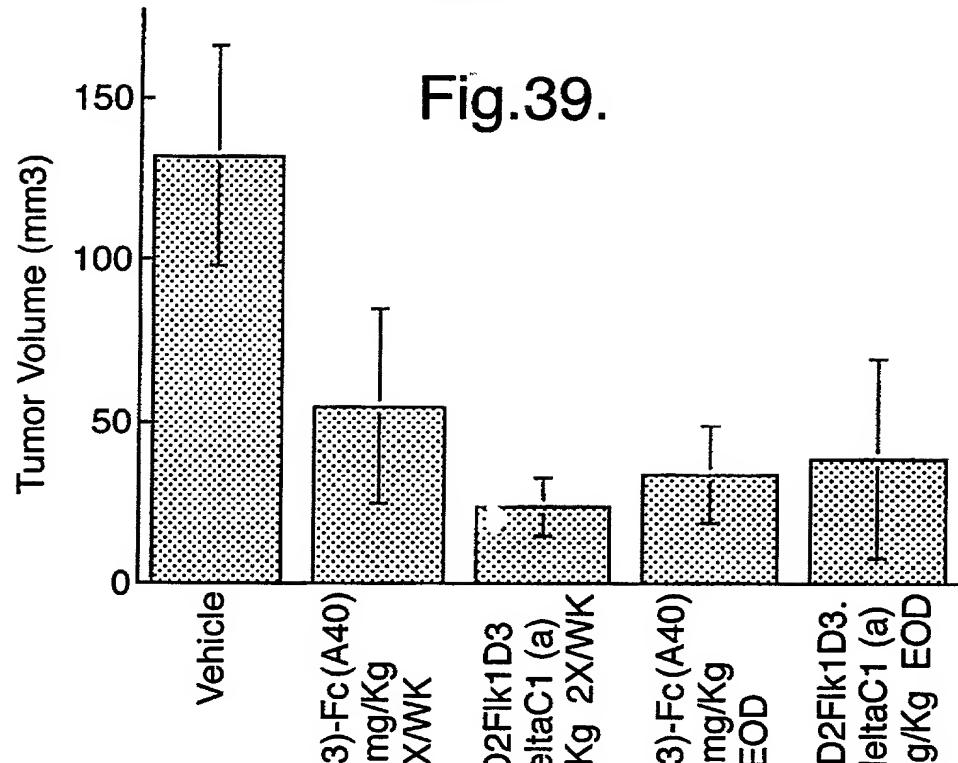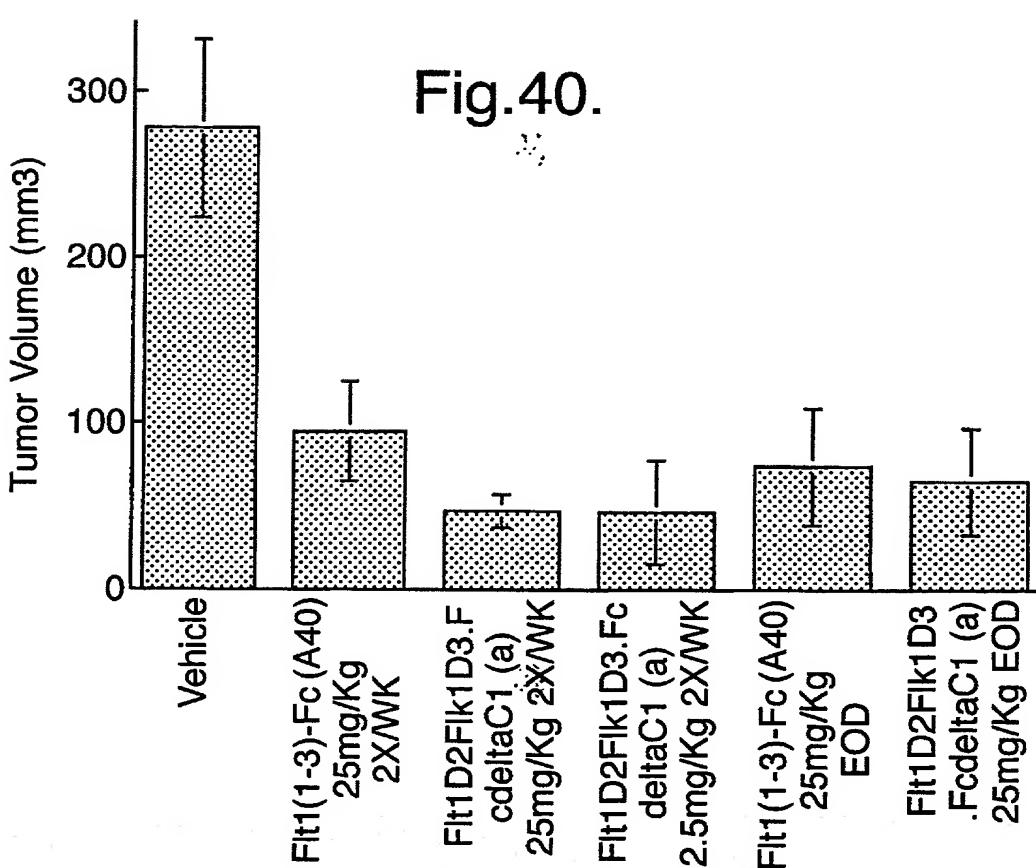
Fig.41.
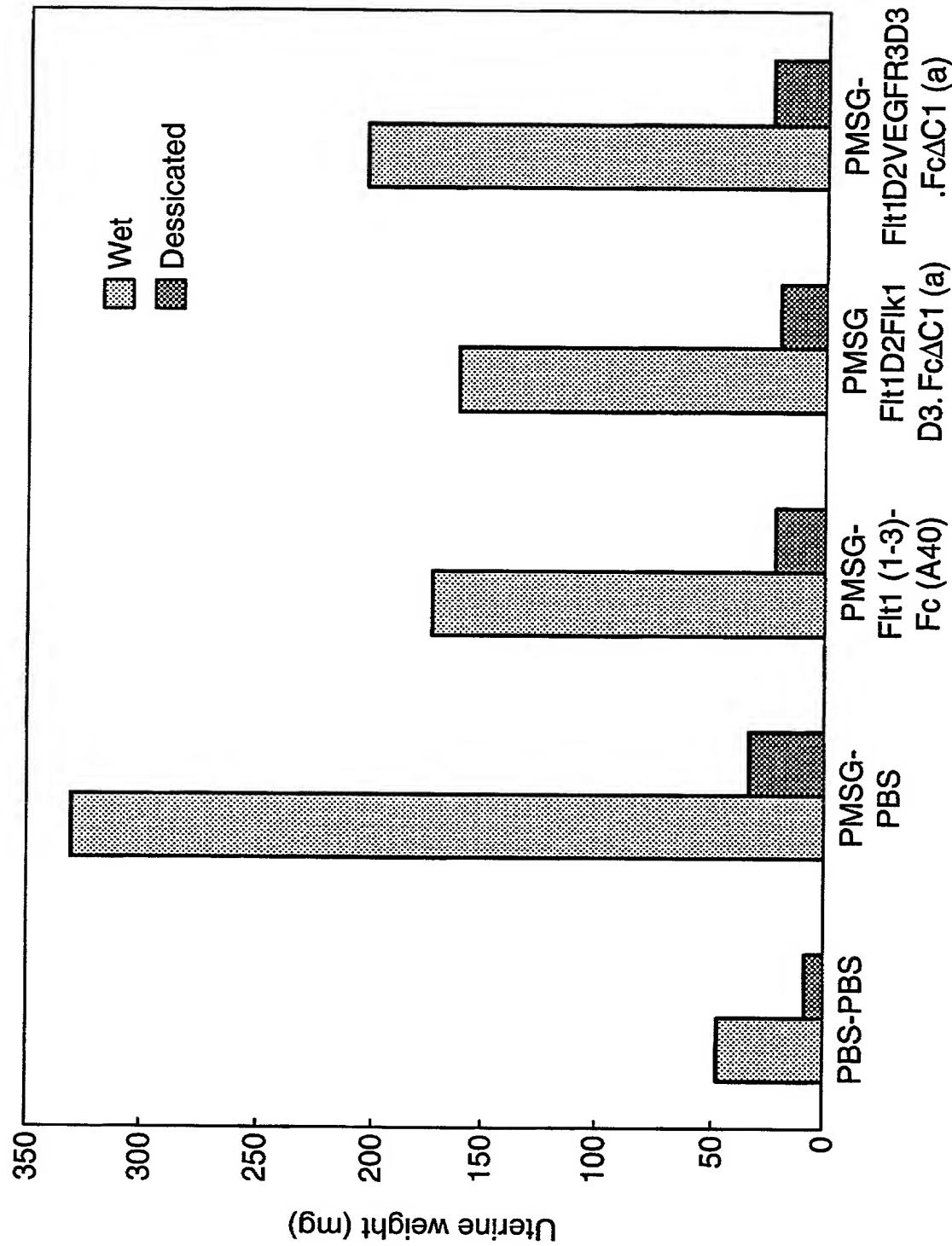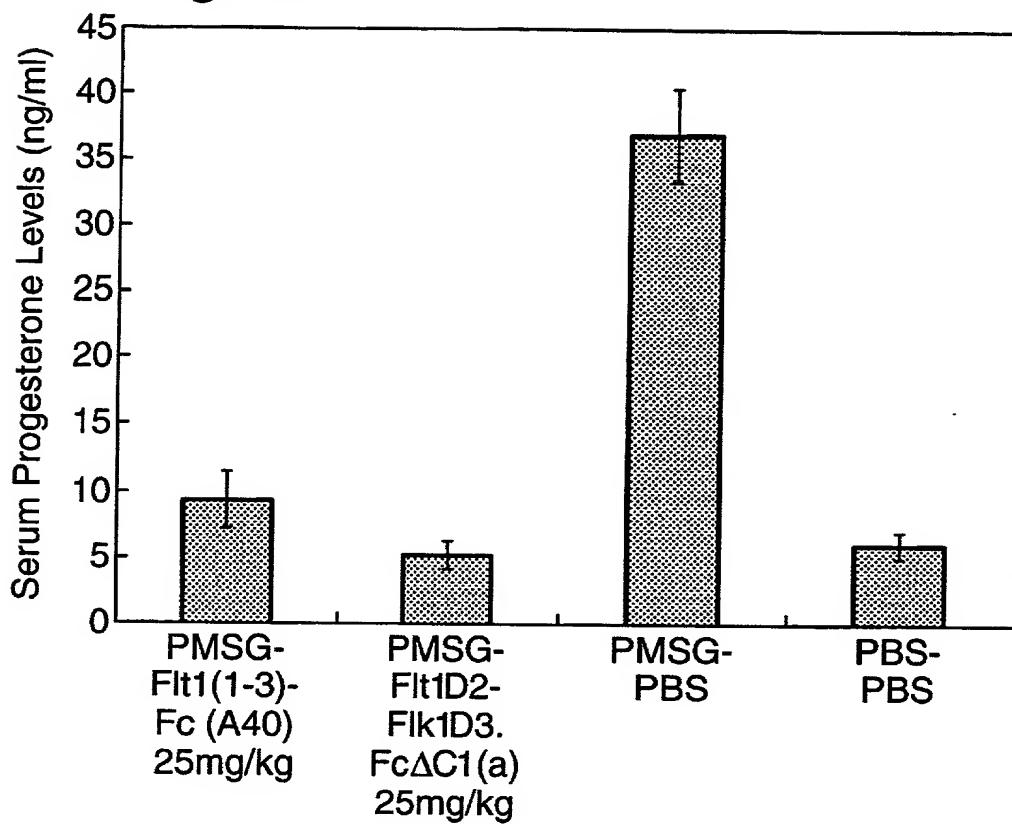
Fig.42A.

Fig.42B.
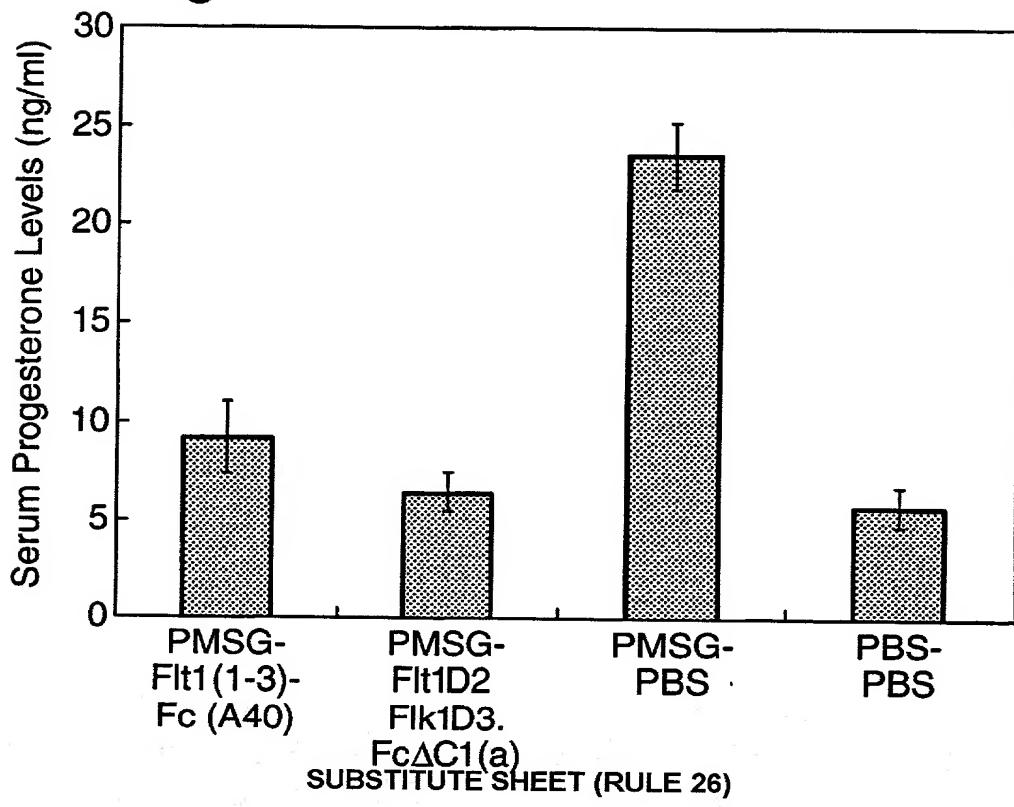
DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled **MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF**, which is the United States national stage filing of International Application PCT/US00/14142 filed May 23, 2000.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

I acknowledge the duty to disclose information of which I am aware that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US00/14142 filed May 23, 2000

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) that occurred between the filing date of the prior application and the national or PCT international filing date of this application:

USSN 60/138,133 filed June 8, 1999

And I hereby appoint Joseph M. Sorrentino (Registration No. 32,598), Gail M. Kempler (Registration No. 32,143), and Linda O. Palladino (Registration No. 45,636) each of them my attorneys and agent, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International

Att. Docket No.REG 710-A-US
USSN: Not Yet Known
US File Date: Filed Herewith
Int'l File No.: PCT/US00/14142
Int'l File Date: May 23, 2000
Declaration and Power of Attorney
Page 2

Applications that are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

> Linda O. Palladino
> Regeneron Pharmaceuticals, Inc.
> 777 Old Saw Mill River Road
> Tarrytown, New York 10591
> Tel. (914-345-7400)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor: __NICHOLAS J. PAPADOPOULOS__
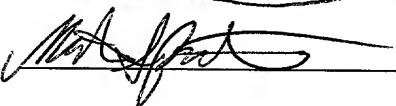
Signature: _____   Date: __12/06/01__

Citizenship: __United States of America__

Residence: __59 Heritage Lane__   N Y

__Lagrangeville, New York 12540__

_____

Post Office Address: same as residence

Att. Docket No.REG 710-A-US
USSN: Not Yet Known
US File Date: Filed Herewith
Int'l File No.: PCT/US00/14142
Int'l File Date: May 23, 2000
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Page 3

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Inventor: <u>GEORGE D. YANCOPOULOS</u>

Signature: _____   Date: _12-6-01_

Citizenship: <u>United States of America</u>

Residence: <u>1519 Baptist Church Road</u> NY

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Post Office Address: same as residence

SEQUENCE LISTING

<110> Nicholas J. Papadopoulos et al.

<120> MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED
PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING
AND USING THEREOF

<130> REG 710-A-US

<140> Not Yet Known
<141> Filed Herewith

<150> PCT/US00/14142
<151> 2000-05-23

<150> 60/138,133
<151> 1999-06-08

<160> 38

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 1704
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1701)

<400> 1

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
                20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca     144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
            35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct     192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
        50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc     240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca     288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta     336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
                100                 105                 110
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cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att      384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115             120             125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa      432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
        130             135             140

att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt      480
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145             150             155             160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act      528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165             170             175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc      576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
        180             185             190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa      624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195             200             205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga      672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
        210             215             220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc      720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225             230             235             240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act      768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
            245             250             255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa aaa      816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
            260             265             270

aat aag aga gct tcc gta agg cga cga att gac caa agc aat tcc cat      864
Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
            275             280             285

gcc aac ata ttc tac agt gtt ctt act att gac aaa atg cag aac aaa      912
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
        290             295             300

gac aaa gga ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa      960
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305             310             315             320

tct gtt aac acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag     1008
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325             330             335

ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct     1056
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
        340             345             350
```

2

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gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag    1104
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        355             360             365

gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg    1152
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    370             375             380

gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac    1200
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385             390             395             400

ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac    1248
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405             410             415

aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac    1296
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420             425             430

tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc    1344
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
        435             440             445

cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga    1392
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    450             455             460

gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag    1440
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465             470             475             480

aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac    1488
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
            485             490             495

atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac tac aag    1536
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            500             505             510

acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc    1584
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        515             520             525

aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca    1632
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
        530             535             540

tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag aag agc    1680
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545             550             555             560

ctc tcc ctg tct ccg ggt aaa tga                                    1704
Leu Ser Leu Ser Pro Gly Lys
                565
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<210> 2
<211> 567
<212> PRT
<213> Homo sapiens

3

<400> 2

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
        20                  25                  30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
        35                  40                  45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
    50                  55                  60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100                 105                 110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115                 120                 125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
    130                 135                 140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165                 170                 175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180                 185                 190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195                 200                 205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210                 215                 220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
            260                 265                 270
Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
        275                 280                 285
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
        290                 295                 300
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305                 310                 315                 320
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325                 330                 335
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
            340                 345                 350
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        355                 360                 365
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    370                 375                 380
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385                 390                 395                 400
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405                 410                 415
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420                 425                 430
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
        435                 440                 445
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    450                 455                 460
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
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4

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       465                   470                   475                   480
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                    485                   490                   495
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
                500                   505                   510
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
            515                   520                   525
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
        530                   535                   540
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                   550                   555                   560
Leu Ser Leu Ser Pro Gly Lys
                565


            <210> 3
            <211> 1674
            <212> DNA
            <213> Homo sapiens

            <220>
            <221> CDS
            <222> (1)...(1671)

            <400> 3

atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
                20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca     144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
            35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct     192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
        50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc     240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca     288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta     336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
                100                 105                 110

cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att     384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
            115                 120                 125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa     432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
            130                 135                 140

att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt     480
```

5

```
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145             150             155             160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act       528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
            165             170             175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc       576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180             185             190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa       624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
            195             200             205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga       672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210             215             220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc       720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225             230             235             240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act       768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
            245             250             255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa att       816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile
            260             265             270

gac caa agc aat tcc cat gcc aac ata ttc tac agt gtt ctt act att       864
Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile
            275             280             285

gac aaa atg cag aac aaa gac aaa gga ctt tat act tgt cgt gta agg       912
Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg
            290             295             300

agt gga cca tca ttc aaa tct gtt aac acc tca gtg cat ata tat gat       960
Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp
305             310             315             320

aaa gca ggc ccg ggc gag ccc aaa tct tgt gac aaa act cac aca tgc      1008
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys
            325             330             335

cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc      1056
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu
            340             345             350

ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag      1104
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
            355             360             365

gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag      1152
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
            370             375             380

ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag      1200
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
```

6

```
ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc      1248
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
            405                 410                 415

acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag      1296
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
            420                 425                 430

gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa      1344
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
            435                 440                 445

gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc      1392
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
450                 455                 460

cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa      1440
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
465                 470                 475                 480

ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag      1488
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
            485                 490                 495

ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc      1536
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
            500                 505                 510

tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag      1584
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
            515                 520                 525

cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac      1632
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
            530                 535                 540

cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tga             1674
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
545                 550                 555
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<210> 4
<211> 557
<212> PRT
<213> Homo sapiens

<400> 4
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
            20                  25                  30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
            35                  40                  45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
    50                  55                  60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95
```

Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
                100   105   110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
            115   120   125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
        130   135   140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145   150   155   160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165   170   175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180   185   190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
            195   200   205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
        210   215   220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225   230   235   240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245   250   255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile
                260   265   270
Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile
            275   280   285
Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg
        290   295   300
Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp
305   310   315   320
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys
            325   330   335
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu
            340   345   350
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
            355   360   365
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
        370   375   380
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
385   390   395   400
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
            405   410   415
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
            420   425   430
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
            435   440   445
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
        450   455   460
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
465   470   475   480
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
                485   490   495
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
            500   505   510
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
            515   520   525
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
        530   535   540
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
545   550   555

<210> 5
<211> 1359

<400> 5

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15

tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc gta gag      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
                20                  25                  30

atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga agg gag     144
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
                35                  40                  45

ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt act tta     192
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
            50                  55                  60

aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc ata atc     240
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80

tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac aaa gaa     288
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95

ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg tat aag     336
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
                100                 105                 110

aca aac tat ctc aca cat cga caa acc aat aca atc ata gat gtc caa     384
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
                115                 120                 125

ata agc aca cca cgc cca gtc aaa tta ctt aga ggc cat act ctt gtc     432
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
                130                 135                 140

ctc aat tgt act gct acc act ccc ttg aac acg aga gtt caa atg acc     480
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160

tgg agt tac cct gat gaa att gac caa agc aat tcc cat gcc aac ata     528
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile
                165                 170                 175

ttc tac agt gtt ctt act att gac aaa atg cag aac aaa gac aaa gga     576
Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly
                180                 185                 190

ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa tct gtt aac     624
Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn
                195                 200                 205

acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag ccc aaa tct     672
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```
Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser
210               215             220

tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg        720
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225               230             235                 240

ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag gac acc ctc        768
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245             250                 255

atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg gac gtg agc        816
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                260             265                 270

cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac ggc gtg gag        864
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
                275             280                 285

gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac aac agc acg        912
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
290               295             300

tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac tgg ctg aat        960
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305               310             315                 320

ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc ccc       1008
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325             330                 335

atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag       1056
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
                340             345                 350

gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc       1104
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
                355             360                 365

agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg       1152
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
370               375             380

gag tgg gag agc aat ggg cag ccg gag aac aac tac aag acc acg cct       1200
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385               390             395                 400

ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc aag ctc acc       1248
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405             410                 415

gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca tgc tcc gtg       1296
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
                420             425                 430

atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc tcc ctg       1344
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
                435             440                 445

tct ccg ggt aaa tga                                                    1359
Ser Pro Gly Lys
```

10

450

<400> 6

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115                 120                 125
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130                 135                 140
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile
                165                 170                 175
Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly
            180                 185                 190
Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn
        195                 200                 205
Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser
    210                 215                 220
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290                 295                 300
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
        355                 360                 365
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415
```

11

```
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
        420                 425             430
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435             440             445
Ser Pro Gly Lys
    450


    <210> 7
    <211> 1389
    <212> DNA
    <213> Homo sapiens

    <220>
    <221> CDS
    <222> (1)...(1386)

    <400> 7
atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5               10              15

tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc gta gag      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20              25              30

atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga agg gag     144
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35              40              45

ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt act tta     192
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
        50              55              60

aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc ata atc     240
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65              70              75              80

tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac aaa gaa     288
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
            85              90              95

ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg tat aag     336
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100             105             110

aca aac tat ctc aca cat cga caa acc aat aca atc ata gat gtc caa     384
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115             120             125

ata agc aca cca cgc cca gtc aaa tta ctt aga ggc cat act ctt gtc     432
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
        130             135             140

ctc aat tgt act gct acc act ccc ttg aac acg aga gtt caa atg acc     480
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145             150             155             160

tgg agt tac cct gat gaa aaa aat aag aga gct tcc gta agg cga cga     528
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg
            165             170             175
```

12

att gac caa agc aat tcc cat gcc aac ata ttc tac agt gtt ctt act   576
Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr
            180   185   190

att gac aaa atg cag aac aaa gac aaa gga ctt tat act tgt cgt gta   624
Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val
            195   200   205

agg agt gga cca tca ttc aaa tct gtt aac acc tca gtg cat ata tat   672
Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr
210   215   220

gat aaa gca ggc ccg ggc gag ccc aaa tct tgt gac aaa act cac aca   720
Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr
225   230   235   240

tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc   768
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
                  245   250   255

ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct   816
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
            260   265   270

gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc   864
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
            275   280   285

aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca   912
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
            290   295   300

aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc   960
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
305   310   315   320

ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc   1008
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
                  325   330   335

aag gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc   1056
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
            340   345   350

aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca   1104
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
            355   360   365

tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc   1152
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
            370   375   380

aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg   1200
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
385   390   395   400

cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac   1248
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
                  405   410   415

ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg   1296

13

```
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
        420                 425                 430

cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac     1344
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
        435                 440                 445

aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa             1386
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455                 460

tga                                                                  1389
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    <210> 8
    <211> 462
    <212> PRT
    <213> Homo sapiens

    <400> 8
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
  1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115                 120                 125
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130                 135                 140
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg
                165                 170                 175
Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr
            180                 185                 190
Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val
        195                 200                 205
Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr
    210                 215                 220
Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230                 235                 240
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
                245                 250                 255
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
            260                 265                 270
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
        275                 280                 285
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
        290                 295                 300
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
305                 310                 315                 320
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
                325                 330                 335
```

14

```
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
        340                 345                 350
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
        355                 360                 365
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
        370                 375                 380
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
385                 390                 395                 400
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
                405                 410                 415
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
                420                 425                 430
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
        435                 440                 445
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
450                 455                 460


        <210> 9
        <211> 1704
        <212> DNA
        <213> Homo sapiens

        <220>
        <221> CDS
        <222> (1)...(1701)

        <400> 9
atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1                   5                   10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
                20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca     144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
            35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct     192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
        50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc     240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca     288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta     336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
                100                 105                 110

cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att     384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
            115                 120                 125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa     432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
```

15

```
att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt      480
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act      528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                        165                 170                 175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc      576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
                180                 185                 190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa      624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
            195                 200                 205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga      672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
        210                 215                 220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc      720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act      768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa aaa      816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
                260                 265                 270

aat aag aac gct tcc gta agg cga cga att gac caa agc aat tcc cat      864
Asn Lys Asn Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
            275                 280                 285

gcc aac ata ttc tac agt gtt ctt act att gac aaa atg cag aac aaa      912
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
        290                 295                 300

gac aaa gga ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa      960
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305                 310                 315                 320

tct gtt aac acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag     1008
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325                 330                 335

ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct     1056
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
                340                 345                 350

gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag     1104
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
                355                 360                 365

gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg     1152
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
                370                 375                 380
```

```
gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac      1200
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385             390             395             400

ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac      1248
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405             410             415

aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac      1296
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420             425             430

tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc      1344
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
            435             440             445

cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga      1392
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
        450             455             460

gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag      1440
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465             470             475             480

aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac      1488
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                485             490             495

atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac tac aag      1536
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
                500             505             510

acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc      1584
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        515             520             525

aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca      1632
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530             535             540

tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag aag agc      1680
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545             550             555             560

ctc tcc ctg tct ccg ggt aaa tga                                      1704
Leu Ser Leu Ser Pro Gly Lys
            565


        <210> 10
        <211> 567
        <212> PRT
        <213> Homo sapiens

        <400> 10
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5               10              15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
                20              25              30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
```

17

```
              35                    40                    45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
     50                    55                    60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                    70                    75                    80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                 85                    90                    95
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
                100                   105                   110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
                115                   120                   125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
     130                   135                   140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                   150                   155                   160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                 165                   170                   175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
                180                   185                   190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
                195                   200                   205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
     210                   215                   220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                   230                   235                   240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                   250                   255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
                260                   265                   270
Asn Lys Asn Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
                275                   280                   285
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
     290                   295                   300
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305                   310                   315                   320
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325                   330                   335
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
                340                   345                   350
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
     355                   360                   365
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
     370                   375                   380
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385                   390                   395                   400
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405                   410                   415
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
                420                   425                   430
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                435                   440                   445
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
     450                   455                   460
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465                   470                   475                   480
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                485                   490                   495
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
                500                   505                   510
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
                515                   520                   525
```

18

```
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530                 535                 540
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                 550                 555                 560
Leu Ser Leu Ser Pro Gly Lys
                565


    <210> 11
    <211> 1453
    <212> DNA
    <213> Homo sapiens


    <220>
    <221> CDS
    <222> (69)...(1442)


    <400> 11
aagcttgggc tgcaggtcga tcgactctag aggatcgatc cccgggcgag ctcgaattcg      60
caaccacc atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg      110
        Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu
          1               5                  10

ctc agc tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc      158
Leu Ser Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe
 15              20                  25                  30

gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga      206
Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly
             35                  40                  45

agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt      254
Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val
         50                  55                  60

act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc      302
Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg
             65                  70                  75

ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac      350
Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr
     80                  85                  90

aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg      398
Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu
 95              100                 105                 110

tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata gat      446
Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp
                 115                 120                 125

gtg gtt ctg agt ccg tct cat gga att gaa cta tct gtt gga gaa aag      494
Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys
             130                 135                 140

ctt gtc tta aat tgt aca gca aga act gaa cta aat gtg ggg att gac      542
Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp
         145                 150                 155

ttc aac tgg gaa tac cct tct tcg aag cat cag cat aag aaa ctt gta      590
Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val
     160                 165                 170
```

19

```
aac cga gac cta aaa acc cag tct ggg agt gag atg aag aaa ttt ttg          638
Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu
175             180             185             190

agc acc tta act ata gat ggt gta acc cgg agt gac caa gga ttg tac          686
Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr
                195             200             205

acc tgt gca gca tcc agt ggg ctg atg acc aag aag aac agc aca ttt          734
Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe
            210             215             220

gtc agg gtc cat gaa aag ggc ccg ggc gac aaa act cac aca tgc cca          782
Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro
            225             230             235

ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc          830
Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe
            240             245             250

ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc          878
Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
255             260             265             270

aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc          926
Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
                275             280             285

aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg          974
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
                290             295             300

cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc          1022
Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr
            305             310             315

gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc          1070
Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
            320             325             330

tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc          1118
Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala
335             340             345             350

aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg          1166
Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
                355             360             365

gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc          1214
Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
            370             375             380

ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg          1262
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
            385             390             395

gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc          1310
Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
    400             405             410
```

20

```
ttc ttc ctc tat agc aag ctc acc gtg gac aag agc agg tgg cag cag      1358
Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
415             420             425             430

ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac      1406
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
                435             440             445

tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tgagcggccg          1452
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                450             455

c                                                                     1453
```

```
        <210> 12
        <211> 458
        <212> PRT
        <213> Homo sapiens

        <400> 12
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5               10              15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20              25              30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35              40              45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50              55              60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65              70              75              80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
            85              90              95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
        100             105             110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Val
        115             120             125
Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val
    130             135             140
Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn
145             150             155             160
Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val Asn Arg
                165             170             175
Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu Ser Thr
            180             185             190
Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr Thr Cys
        195             200             205
Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg
    210             215             220
Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys
225             230             235             240
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                245             250             255
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
            260             265             270
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
        275             280             285
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
        290             295             300
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305             310             315             320
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
```

21

```
                      325                  330                  335
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340                  345                  350
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                  360                  365
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                  375                  380
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                  390                  395                  400
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                 405                  410                  415
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                  425                  430
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                  440                  445
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                  455
```

```
<210> 13
<211> 1444
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (69)...(1433)

<400> 13
aagcttgggc tgcaggtcga tcgactctag aggatcgatc cccgggcgag ctcgaattcg    60
caaccacc atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg   110
         Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu
          1               5                      10

ctc agc tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc   158
Leu Ser Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe
 15                  20                  25                  30

gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga   206
Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly
                 35                  40                  45

agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt   254
Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val
             50                  55                  60

act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc   302
Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg
         65                  70                  75

ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac   350
Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr
     80                  85                  90

aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg   398
Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu
 95                  100                 105                 110

tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata gat   446
Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp
                 115                 120                 125
```

22

```
atc cag ctg ttg ccc agg aag tcg ctg gag ctg ctg gta ggg gag aag          494
Ile Gln Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys
            130             135             140

ctg gtc ctc aac tgc acc gtg tgg gct gag ttt aac tca ggt gtc acc          542
Leu Val Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr
            145             150             155

ttt gac tgg gac tac cca ggg aag cag gca gag cgg ggt aag tgg gtg          590
Phe Asp Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val
    160             165             170

ccc gag cga cgc tcc caa cag acc cac aca gaa ctc tcc agc atc ctg          638
Pro Glu Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu
175             180             185             190

acc atc cac aac gtc agc cag cac gac ctg ggc tcg tat gtg tgc aag          686
Thr Ile His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys
            195             200             205

gcc aac aac ggc atc cag cga ttt cgg gag agc acc gag gtc att gtg          734
Ala Asn Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val
            210             215             220

cat gaa aat ggc ccg ggc gac aaa act cac aca tgc cca ccg tgc cca          782
His Glu Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro
            225             230             235

gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa          830
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
            240             245             250

ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg          878
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
255             260             265             270

gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac          926
Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
            275             280             285

gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag          974
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
            290             295             300

cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac          1022
Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
            305             310             315

cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa          1070
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
            320             325             330

gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag          1118
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
335             340             345             350

ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg          1166
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu
            355             360             365

acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc          1214
```

23

```
Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
            370             375                 380

agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac   1262
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
            385             390                 395

tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc   1310
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
            400             405                 410

tat agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc   1358
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val
415             420                 425                 430

ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag   1406
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln
            435             440                 445

aag agc ctc tcc ctg tct ccg ggt aaa tgagcggccg c                  1444
Lys Ser Leu Ser Leu Ser Pro Gly Lys
            450                 455


        <210> 14
        <211> 455
        <212> PRT
        <213> Homo sapiens


        <400> 14
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
            35                  40                  45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
            50                  55                  60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Ile Gln
            115                 120                 125
Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys Leu Val
            130                 135                 140
Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr Phe Asp
145                 150                 155                 160
Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val Pro Glu
                165                 170                 175
Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu Thr Ile
            180                 185                 190
His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys Ala Asn
            195                 200                 205
Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val His Glu
            210                 215                 220
Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
225                 230                 235                 240
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
```

```
                    245                       250                       255
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
            260                       265                       270
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
        275                       280                       285
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
    290                       295                       300
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305                       310                       315                   320
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                325                       330                       335
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
            340                       345                       350
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
        355                       360                       365
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
    370                       375                       380
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
385                       390                       395                   400
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
                405                       410                       415
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
            420                       425                       430
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
            435                       440                       445
Leu Ser Leu Ser Pro Gly Lys
450                       455
```

```
<210> 15
<211> 1377
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1374)

<400> 15
atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc        48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5                   10                  15

tgt ctg ctt ctc aca gga tct agt tcc gga agt gat acc ggt aga cct        96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Asp Thr Gly Arg Pro
                20                  25                  30

ttc gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa       144
Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu
            35                  40                  45

gga agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act       192
Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr
        50                  55                  60

gtt act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa       240
Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys
65                  70                  75                  80

cgc ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg       288
Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr
                85                  90                  95
```

25

```
tac aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat      336
Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His
            100                 105                 110

ttg tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata      384
Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile
            115                 120                 125

gat gtg gtt ctg agt ccg tct cat gga att gaa cta tct gtt gga gaa      432
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
            130                 135                 140

aag ctt gtc tta aat tgt aca gca aga act gaa cta aat gtg ggg att      480
Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
145                 150                 155                 160

gac ttc aac tgg gaa tac cct tct tcg aag cat cag cat aag aaa ctt      528
Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
                165                 170                 175

gta aac cga gac cta aaa acc cag tct ggg agt gag atg aag aaa ttt      576
Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
                180                 185                 190

ttg agc acc tta act ata gat ggt gta acc cgg agt gac caa gga ttg      624
Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
            195                 200                 205

tac acc tgt gca gca tcc agt ggg ctg atg acc aag aag aac agc aca      672
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
            210                 215                 220

ttt gtc agg gtc cat gaa aag gac aaa act cac aca tgc cca ccg tgc      720
Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys
225                 230                 235                 240

cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca      768
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                245                 250                 255

aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc      816
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
                260                 265                 270

gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg      864
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
            275                 280                 285

tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag      912
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
            290                 295                 300

gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg      960
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305                 310                 315                 320

cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac     1008
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
                325                 330                 335
```

26

```
aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg      1056
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340             345             350

cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag      1104
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
            355             360             365

ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat      1152
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
        370             375             380

ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac      1200
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385             390             395             400

aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc      1248
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405             410             415

ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac      1296
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420             425             430

gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg      1344
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
            435             440             445

cag aag agc ctc tcc ctg tct ccg ggt aaa tga                          1377
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
        450             455
```

```
<210> 16
<211> 458
<212> PRT
<213> Homo sapiens

<400> 16
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1               5               10              15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Asp Thr Gly Arg Pro
            20              25              30
Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu
            35              40              45
Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr
        50              55              60
Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys
65              70              75              80
Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr
                85              90              95
Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His
            100             105             110
Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile
            115             120             125
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
        130             135             140
Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
145             150             155             160
Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
                165             170             175
```

27

Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
              180   185   190
Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
              195   200   205
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
    210   215   220
Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys
225   230   235   240
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                  245   250   255
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
              260   265   270
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
              275   280   285
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
    290   295   300
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305   310   315   320
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
                  325   330   335
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
              340   345   350
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
              355   360   365
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370   375   380
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385   390   395   400
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                  405   410   415
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
              420   425   430
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
              435   440   445
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450   455

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        <213> Homo sapiens

        <400> 17
Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His
1   5   10   15
Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro
              20   25   30
Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro
              35   40   45
Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser
    50   55   60
Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val
65   70   75   80
Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn
                  85   90   95
Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser
              100   105   110
Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn
              115   120   125
Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His
              130   135   140

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Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met
145             150             155             160
Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp
            165             170             175
Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys
            180             185             190
Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr
            195             200             205
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser
    210             215                 220
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
225             230             235             240
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro
            245             250             255
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
            260             265             270
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val
            275             280             285
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
            290             295             300
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
305             310             315             320
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
            325             330             335
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
            340             345             350
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
            355             360             365
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
    370             375             380
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
385             390             395             400
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
            405             410             415
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Pro Gly Lys
            420             425             430

            <210> 18
            <211> 36
            <212> DNA
            <213> Artificial Sequence

            <220>
            <223> primer

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gactagcagt ccggaggtag acctttcgta gagatg                                    36

            <210> 19
            <211> 33
            <212> DNA
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            <220>
            <223> primer

            <400> 19
cggactcaga accacatcta tgattgtatt ggt                                       33

            <210> 20
            <211> 7
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            <212> PRT
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            <400> 20
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         1                   5


            <210> 21
            <211> 35
            <212> DNA
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            <220>
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            <400> 21
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            <210> 22
            <211> 38
            <212> DNA
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            <220>
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            <400> 22
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            <210> 23
            <211> 6
            <212> PRT
            <213> Homo sapiens

            <400> 23
        Val Arg Val His Glu Lys
         1                   5


            <210> 24
            <211> 36
            <212> DNA
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            <220>
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            <400> 24
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            <210> 25
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            <220>
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Ile Gln Leu Leu
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         <212> DNA
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atccagctgt tgcccaggaa gtcgctggag ctgctggta                          39


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         <211> 39
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         <400> 28
attttcatgc acaatgacct cggtgctctc ccgaaatcg                          39


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         <212> DNA
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         <220>
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         <213> Artificial Sequence

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gataatgccc gggccatttt catgcacaat gacctcggt                          39


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         <213> Homo sapiens

         <400> 31
Val Ile Val His Glu Asn
 1               5
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<210> 32
<211> 10
<212> PRT
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<220>
<223> modified Flt1 receptor

<400> 32
Lys Asn Lys Arg Ala Ser Val Arg Arg Arg
1   5   10

<210> 33
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<212> PRT
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<220>
<223> modified Flt1 receptor

<400> 33
Asn Ala Ser Val Asn Gly Ser Arg
1   5

<210> 34
<211> 10
<212> PRT
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<220>
<223> modified Flt1 receptor

<400> 34
Lys Asn Lys Cys Ala Ser Val Arg Arg Arg
1   5   10

<210> 35
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<212> PRT
<213> Homo sapiens

<400> 35
Ser Lys Leu Lys
1

<210> 36
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<400> 36
Lys Asn Lys Arg Ala Ser Val Arg Arg
1   5

<210> 37
<211> 4
<212> PRT
<213> Homo sapiens

<400> 37

Thr Ile Ile Asp
  1

        <210> 38
        <211> 4
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        <213> Homo sapiens

        <400> 38

Val Val Leu Ser
  1